Supplementary Information

Millimeter-Scale Contact Printing of Aqueous Solutions using a Stamp Made out of Paper and Tape

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Fabrication of Paper-based Stamp

Paper-based microfluidic channels

We patterned chromatography paper (Whatman No. 1) by photolithography as described previously.[1-3] Paper was impregnated with SU-8 photoresist, baked on a hot plate for 10 minutes at 110 °C, cooled to room temperature and exposed to UV light (IntelliRay 600, UVitron International, Inc.) for 14 s with an intensity of 100 mW/cm^2 through a transparency mask. The paper was then baked a second time for 5 minutes at 110 °C and cooled to room temperature. The patterns were developed in a bath of acetone for 1 minute followed by a rinse in acetone and a rinse in 70% isopropyl alcohol. The paper was blotted between two paper towels, rinsed a second time with 70% isopropyl alcohol, blotted, and allowed to dry under ambient conditions.

Laser cutting of tape

Double-sided adhesive tape (ACE plastic carpet tape 50106) was patterned with holes using a laser cutter (Universal Laser VL-300 50 Watt Versa Laser). The tape was placed on a sheet of parchment paper to protect the adhesive during the cutting process.

Assembly of paper-based stamp

We assembled the devices by stacking alternating layers of paper and double-sided tape from the bottom up.[2] The alignment of holes and channels between layers was achieved by a manual process with an accuracy of ~500 μm. The bottom face of the double-sided tape was first attached to the bottom layer of the device. The top face of the double-sided tape remained protected by the plastic backing supplied with the tape. The holes in the tape were filled with a paste made from a mixture of cellulose powder and water (1:3 w/w cellulose powder–water). Excess paste was scraped off of the plastic backing using a

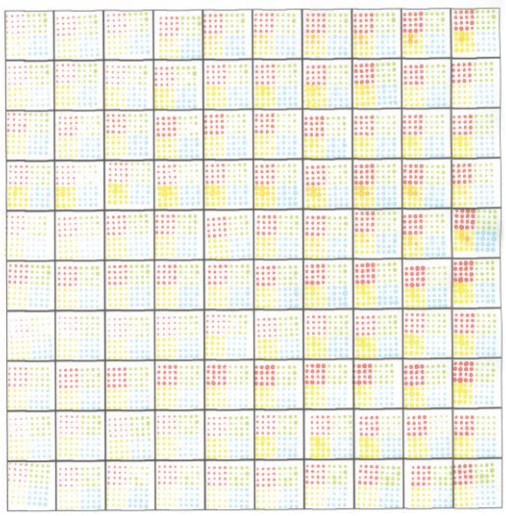
metal spatula, and the plastic backing was peeled from the tape. The top layer of paper was attached to the tape, and the entire device was compressed with a manual press (61401 AP40 Arbor Press, Palmgren, Inc.) under pressure of approximately 20 kg/cm^2 to achieve conformal contact between paste and paper. Additional layers of paper and tape were then added using the same process to complete the device.

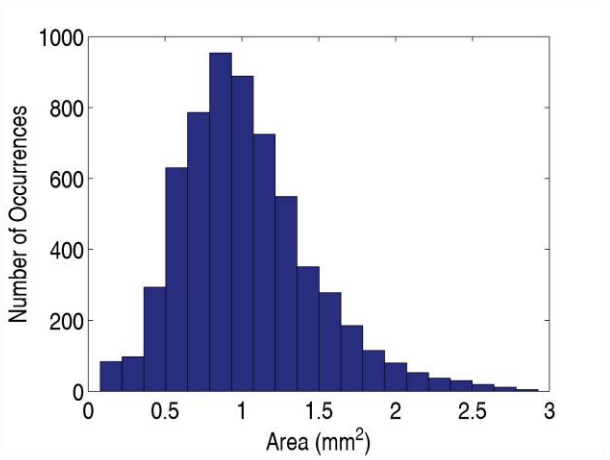
Supplementary Figure 1. This figure shows 10 × 10 grids of 64 spots (6400 total spots) with a nominal diameter of 1 mm (area of 0.8 mm^2) scanned at 600 dpi. For the image shown in (A), the mean of 6166 measured spots with areas between 0.08 mm^2 and 3.1 mm^2 is 1.0 ± 0.4 mm^2. For the image shown in (B), the mean of 6394 measured spots with areas between 0.08 mm^2 and 3.1 mm^2 is also 1.0 ± 0.4 mm^2. To the right of the scanned images are histograms depicting the distributions for measured areas of the spots. A customized algorithm (included below) in MATLAB counted the number of connected pixels in each spot within an image to determine areas for the spots. Calculating the standard deviation between the three 8-bit intensities for each RGB pixel in the images and comparing the standard deviation at each pixel to a set threshold with a value of 15, the algorithm selected pixels pertaining to the red, green, yellow, and blue printed spots. Then, the algorithm formed regions of adjacent pixels selected in the prior step and calculated the areas of these selected regions. If the calculated area of an individual region was greater than 10% of the nominal area of the printed circles (0.08 mm^2) and less than 400% of the nominal area (3.1 mm^2), the compiled histogram included the calculated area for the individual spot. This range of permissible areas for depicting the distribution limited the counting of spurious, highlighted areas not pertaining to printed spots and also eliminated the counting of some of the spots, which had merged with each other. The histogram in (A) includes measurements for 6166 spots but does not include 810 small clumps of pixels each with an area less than 0.08 mm^2. Of these 810 small clumps, 376 are single pixels and another 136 are pairs of pixels. The histogram in (A) also does not include 53 large clumps of pixels pertaining to large clumps of spots, which had spread into each other. The histogram in (B) includes

measurements for 6394 spots but does not include 850 small clumps of pixels with areas less than 0.08 mm^2, nor 2 clumps with measured areas greater than 3.1 mm^2. While this algorithm may not be as accurate as other potential methods, it is fast (required less than a minute on a laptop to process tagged image files of size greater than 50 Mbytes) and simple (fully described in just a few sentences). For the large 8-bit images greater than 50 Mbytes in size, the algorithm separated the images into sub-images to avoid "out of memory" errors in MATLAB.

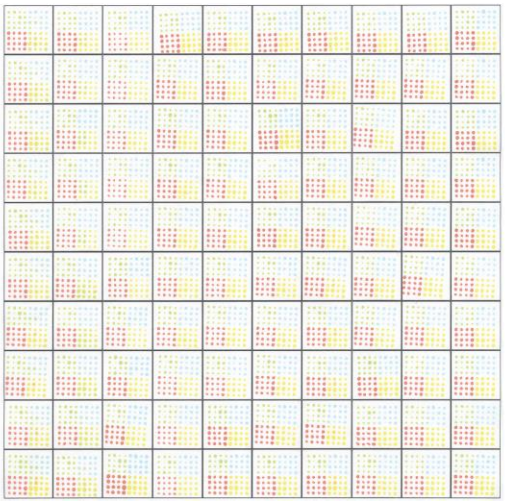
Supplementary Figure 1.

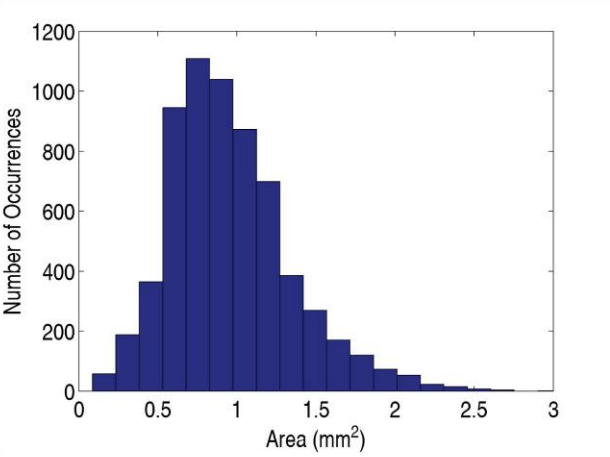
A)





B)





MATLAB Script for Image Analysis of Colored Spots

```
% This MATLAB script analyzes images with colored spots and returns
% histograms for the distributions of diameters and areas of the spots.
% The variable ImageName is set to run with the data file 1-1.tif.
%% Initialization
clear all;
close all;
clc
%% Load image
ImageName='1-1.tif';
[Image,ImageMap]=imread(ImageName,'tif');
%% Pixels to area
ScannerDPI=600;  %dots/inch
PixelConv=1/ScannerDPI*25.4;
PixelConvSquared=PixelConv^2; %mm^2/pixel
DefinedDotDiameter=1; %mm
DefinedDotArea=pi*(DefinedDotDiameter/2)^2; %mm^2
DefinedDotPixels=pi*(DefinedDotDiameter/PixelConv/2)^2; %Area in pixels
%% Split into 4 regions because of the size of the image
ImageSize=size(Image);
XSize=ImageSize(2);
YSize=ImageSize(1);
SetCenterPoint=[2170 2254];
Region(1).Image=imcrop(Image,[1 1 SetCenterPoint(1)
SetCenterPoint(2)]);
Region(2).Image=imcrop(Image,[SetCenterPoint(1)+1 1 ...
    XSize-SetCenterPoint(1)-1 SetCenterPoint(2)]);
Region(3).Image=imcrop(Image,[1 SetCenterPoint(2)+1 ...
    SetCenterPoint(1) YSize-SetCenterPoint(2)-1]);
Region(4).Image=imcrop(Image, [SetCenterPoint(1)+1 SetCenterPoint(2)+1
...
    XSize-SetCenterPoint(1)-1 YSize-SetCenterPoint(2)-1]);
%% Show image and select regions of interest
for k=1:length(Region),
    IDouble=double(Region(k).Image)/255;
    StdI=std(IDouble,0,3);
    StdDevThreshold=15;
    Region(k).BW=im2bw(StdI,StdDevThreshold/255);
end
%% Crop Images, Black and White Conversion, and Analysis
for k=1:length(Region),
    figure
    imshow(Region(k).BW)
    title(['Region ',num2str(k)])
    [Region(k).Labeled,Region(k).NumObjects] = bwlabel(Region(k).BW,4);
    PseudoColor=label2rgb(Region(k).Labeled, @spring, 'c', 'shuffle');
    figure
    %imshow(Region(k).Labeled)
    imshow(PseudoColor)
    Region(k).CircleData=regionprops(Region(k).Labeled,'basic');
end
%% Compile Statistics
Counter=0;
for k=1:length(Region),
    for i=1:length(Region(k).CircleData),
        Counter=Counter+1;
```

```
        AllAreas(Counter)=Region(k).CircleData(i).Area;
        %AllTheCentroids=CircleData(i.j).RegionProps(k).Centroids
    end
end
TotalCount=Counter;
MaxArea=4*DefinedDotPixels; %Pixels
MaxAreaMM=MaxArea*PixelConvSquared;
MinArea=0.1*DefinedDotPixels; %Pixels
MinAreaMM=MinArea*PixelConvSquared;
AllAreasLessThanIndices=find(AllAreas<MaxArea);
AllAreasGreaterThanIndices=find(AllAreas>MinArea);
MidRangeIndices=intersect(AllAreasLessThanIndices,...
    AllAreasGreaterThanIndices);
AreasGreaterThan=AllAreas(AllAreasGreaterThanIndices);
AreasLessThan=AllAreas(AllAreasLessThanIndices);
AreasMidRange=AllAreas(MidRangeIndices);
%% Show Statistics
MeanAllAreas=mean(AllAreas);
MeanAllAreasMM=PixelConvSquared*mean(AllAreas);
MeanAreasLessThan=mean(AreasLessThan);
MeanAreasMidRange=mean(AreasMidRange);
MeanAreaGreaterThan=mean(AreasGreaterThan);
MeanAreasLessThanMM=PixelConvSquared*MeanAreasLessThan;
MeanAreasMidRangeMM=PixelConvSquared*MeanAreasMidRange;
MeanAreasGreaterThanMM=PixelConvSquared*MeanAreaGreaterThan;
StdAllAreas=std(AllAreas);
StdAllAreasMM=PixelConvSquared*std(AllAreas);
StdAreasLessThan=std(AreasLessThan);
StdAreasLessThanMM=StdAreasLessThan*PixelConvSquared;
StdAreasMidRange=std(AreasMidRange);
StdAreasMidRangeMM=StdAreasMidRange*PixelConvSquared;
StdAreasGreaterThan=std(AreasGreaterThan);
StdAreasGreaterThanMM=PixelConvSquared*StdAreasGreaterThan;
DiametersMidRange=sqrt(4/pi*AreasMidRange);
MeanDiameterMidRange=mean(DiametersMidRange)
MeanDiameterMidRangeMM=PixelConv*MeanDiameterMidRange
StdDiameter=std(DiametersMidRange)
StdDiameterMidRangeMM=PixelConv*StdDiameter
N=length(AllAreas);
NLess=length(AreasLessThan)
NMidRange=length(AreasMidRange)
NGreater=length(AreasGreaterThan)
NLeft=N-NGreater;
NRight=N-NLess;
figure
hist(AllAreas*PixelConvSquared,200)
text(4,700,'All Areas')
text(4,600,['N=',num2str(N)])
text(4,500,['Mean=',num2str(MeanAllAreasMM,4),' mm^2'])
text(4,400,['Std Dev=',num2str(StdAllAreasMM,3),' mm^2'])
text(18,700,['Area < ',num2str(MaxAreaMM,3),' mm^2'])
text(18,600,['N=',num2str(NLess)])
text(18,500,['Mean=',num2str(MeanAreasLessThanMM,4),' mm^2'])
text(18,400,['Std Dev=',num2str(StdAreasLessThanMM,3),' mm^2'])
text(32,700,['Area > ',num2str(MinAreaMM,3),' mm^2'])
text(32,600,['N=',num2str(NGreater)])
text(32,500,['Mean=',num2str(MeanAreasGreaterThanMM,4),' mm^2'])
```

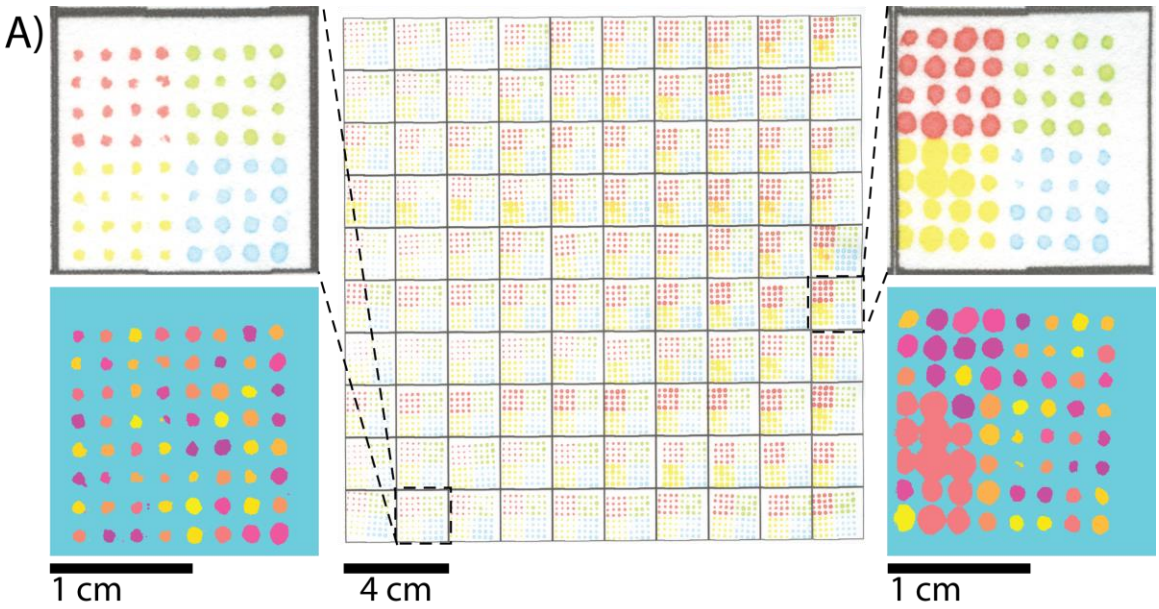
```
text(32,400,['Std Dev=',num2str(StdAreasGreaterThanMM,3),' mm^2'])
xlabel('Area (mm^2)','FontSize',14)
ylabel('Number of Occurrences','FontSize',14)
set(gca,'FontSize',12)
figure
hist(AreasLessThan,20)
xlabel('Area (pixels)')
ylabel('Number of Occurrences')
figure
hist(AreasMidRange,20)
xlabel('Area (pixels)')
ylabel('Number of Occurrences')
text(70,800,['N=',num2str(NLess)])
text(70,700,['Mean=',num2str(MeanAreasLessThan,4)])
text(70,600,['Std Dev=',num2str(StdAreasLessThan,4)])
figure
hist(AreasLessThan*PixelConvSquared,20)
text(2,800,['N=',num2str(NLess)])
text(2,700,['Mean=',num2str(MeanAreasLessThanMM,4),' mm^2'])
text(2,600,['Std Dev=',num2str(StdAreasLessThanMM,4),' mm^2'])
xlabel('Area (mm^2)')
ylabel('Number of Occurrences')
%% Histogram for Range
figure
hist(AreasMidRange*PixelConvSquared,20)
text(1.6,900,...
    [num2str(MinAreaMM,2),' mm^2 < Area < ',num2str(MaxAreaMM,3),'
mm^2'])
text(1.6,800,['N (in range)=',num2str(NMidRange)])
text(1.6,700,['N(<',num2str(MinAreaMM,2),' mm^2) = ',...
    num2str(NLeft)])
text(1.6,600,['N(>',num2str(MaxAreaMM,3),' mm^2) = ',...
    num2str(NRight)])
text(1.6,500,['Mean=',num2str(MeanAreasMidRangeMM,4),' mm^2'])
text(1.6,400,['Std Dev=',num2str(StdAreasMidRangeMM,3),' mm^2'])
xlabel('Area (mm^2)')
ylabel('Number of Occurrences')
```

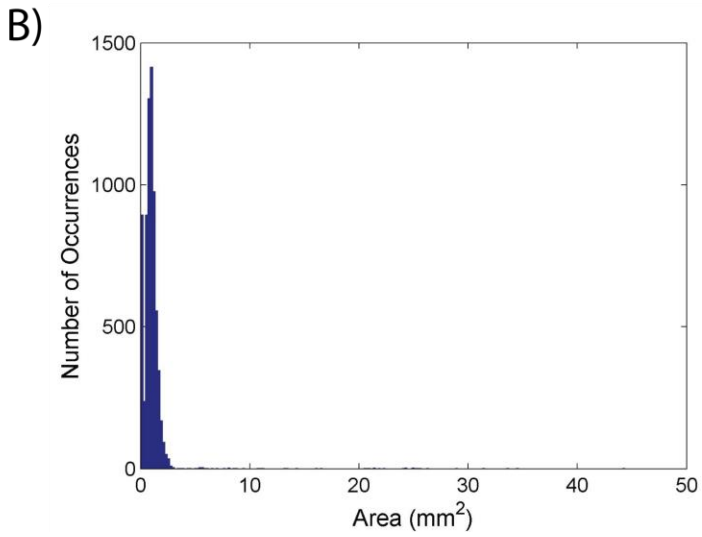
Supplementary Figure 2. In Figure 3, Supplementary Figure 1, Supplementary Figure 3, and Supplementary Figure 6, the algorithm for counting colored spots measured different quantities of spots for patterns printed with the same stamp. The different quantities of measured spots resulted from difficulties with the image processing and variability in the physical process of contact printing. The images in (A) show two magnified sections of the same printed pattern displayed in Supplementary Figure 1A. The upper left image displays a set of printed spots that did not coalesce with each other, and the pseudo-color map on the left shows that the algorithm recognized more than the 64 intentionally printed spots. The counting of more than the 64 spots results from some spurious spots detected with the low threshold (standard deviation of 15 for the three 8-bit intensities for each RGB pixel) and also the visible separation of a printed spot into two parts (4^{th} spot from the top and 4^{th} spot from the left). The upper right image and its corresponding pseudo-colored image exhibit a set of printed spots totalling less than the 64 intentionally printed spots because some of the printed spots coalesced with each other. To assess the mean and standard deviation of the areas of the printed spots given in the manuscript, additional thresholds for permissible areas of counted spots eliminated the extremely small, spurious spots (less than 10% of the nominal area of each spot) and the large clumps of spots that coalesced with each other (greater than 400% of the nominal area of each spot).

The histogram in (B) shows the distribution of all the recognized areas by the algorithm for Supplementary Figure 1A without the specified thresholds on the areas of the spots (0.08 mm^2 to 3.1 mm^2). The mean area for all of the spots is 1.0 ± 1.6 mm^2 with N=7029, the mean area for all of the spots with areas less than 3.1 mm^2 is 0.9 ± 0.5 mm^2

with N=6976, the mean area for all of the spots with areas greater than 0.08 mm^2 is 1.1 ± 1.6 mm^2 with N=6219, and as stated previously, the mean area for the spots with areas between 0.08 mm^2 and 3.1 mm^2 is 1.0 ± 0.4 mm^2 with N=6166. The fraction of useful printed spots will depend on the application. Using the 6166 counted drops within the specified thresholds for area, assuming the spots less than 0.08 mm^2 were spurious data, and assuming that any of the counted spots that might have coalesced but not formed areas greater than 400% of the nominal area (3.1 mm^2), an estimate for the useful percentage of printed spots is 96% (6166 counted drops/6400 nominally printed drops). Going one step further and stating that useful drops must also have an area greater than half the nominal area (0.4 mm^2) and less than 400% of the nominal area (3.1 mm^2), the number of counted drops would be 5947 and the percentage of useful drops would be 93% (5947 counted drops/6400 nominally printed drops).

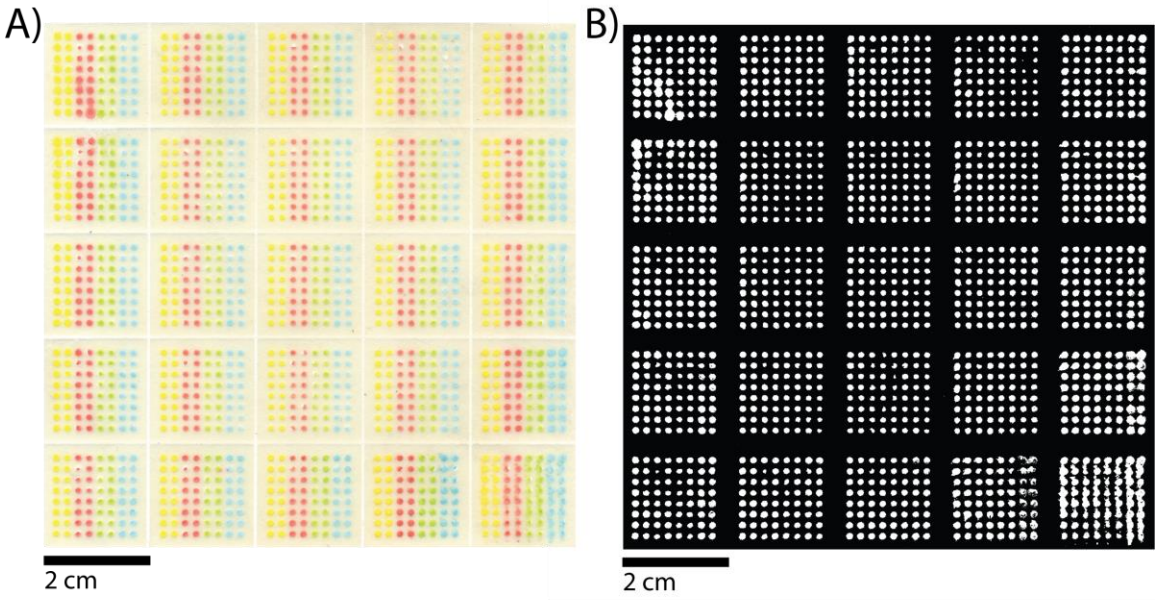
Supplementary Figure 2.

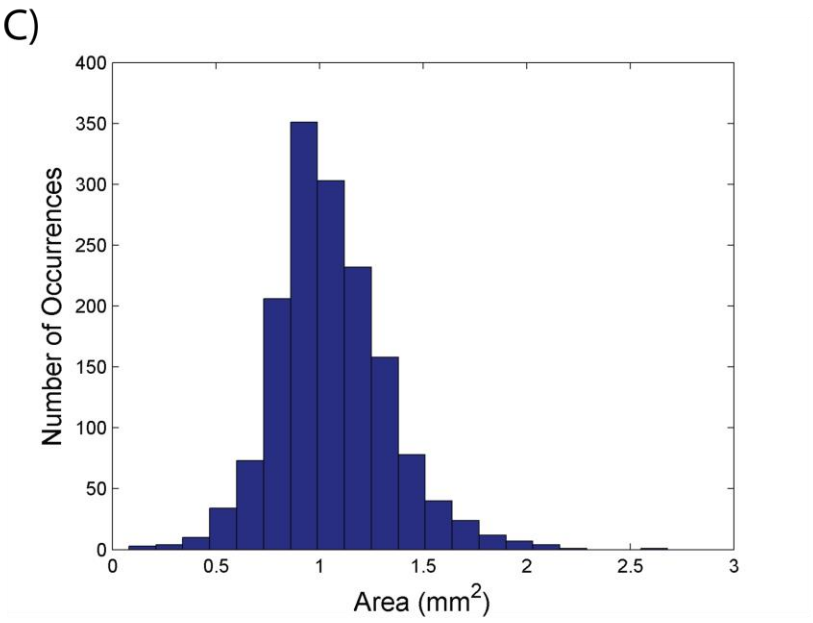




Supplementary Figure 3. This figure shows the results of transferring aqueous solutions from a paper-based stamp to a paper-based substrate pre-patterned using photolithography and SU-8. The image in (A) shows a 5 × 5 grids of 64 spots (1600 total spots) with a nominal diameter of 1 mm (area of 0.8 mm^2) scanned at 600 dpi. The mean of 1541 measured spots with areas between 0.08 mm^2 and 3.1 mm^2 is 1.1 ± 0.3 mm^2 (calculated mean diameter is 1.2 ± 0.2 mm). The image shown in (B) is a black and white image created from (A) using MATLAB and three different thresholds to select the appropriate regions for image processing and statistical analysis. This histogram for the measured areas between 0.08 mm^2 and 3.1 mm^2 is in (C).

Supplementary Figure 3.





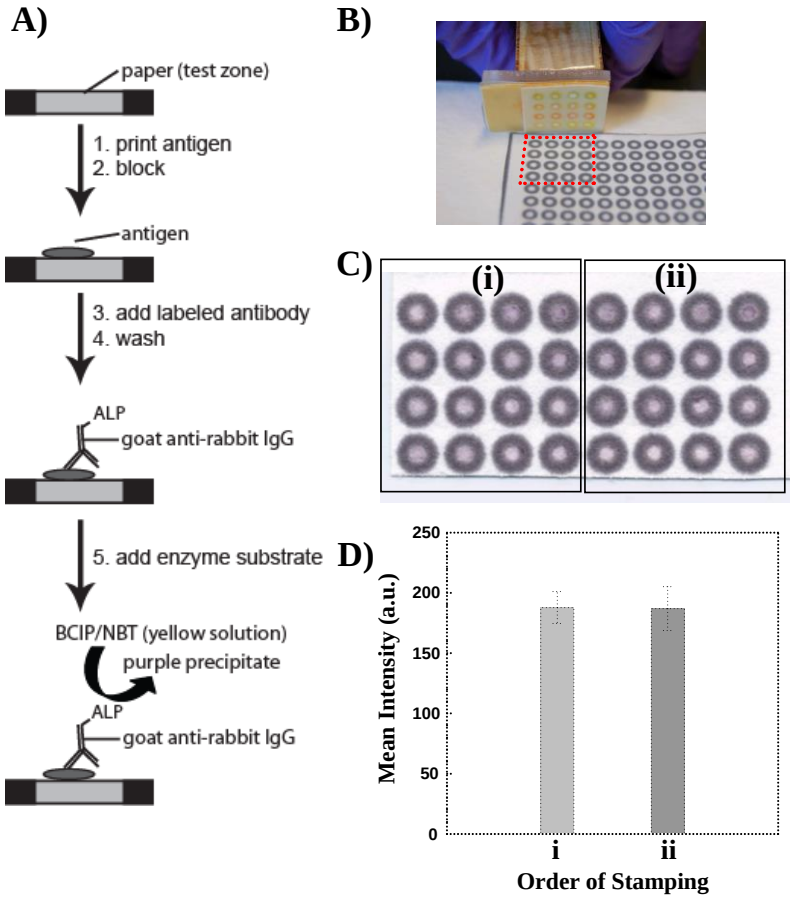
Contact printing-based ELISA

ELISA (enzyme-linked immunosorbent assays) technology is universally used to detect antibodies and antigens.[4,5] We have also carried out high-throughput paper-based enzyme-linked immunosorbent assays (P-ELISA) using the paper-based stamp; Supplementary Figure 4A outlines this process.[6] We used wax printing to create test zones on Whatman Chromatography paper No. 1. (Supplementary Figure 4B); the wax boundary on the paper substrate prevented wicking of the fluid beyond specified regions.[7]

We followed a five-step procedure for indirect P-ELISA using the paper-based stamp: i) We printed 6.7 nM rabbit IgG into test zones (~0.5 µL for each test zone) and then waited for 10 minutes. ii) We treated each test zone with 0.5 µL of blocking buffer[8] to prevent non-specific adsorption of proteins. iii) We then exposed the printed rabbit IgG to 0.5 µL of enzyme-linked antibody per zone.[9] iv) We washed away unbound antibodies, using 2.5-µL aliquots of PBS per zone (three times). v) We finally added the substrate solution (1 µL ALP substrate solution for each test zone).[10] The mean intensity of color was determined by scanning the paper-based test zones (Supplementary Figure 4C) and measuring color in the test zones using ImageJ.[11] Thirty-two test zones (6.7 nM rabbit IgG) were obtained by two printing steps (16 test zones for each). The intensities in the test zones (32 test zones for 2 groups) formed in these two tests were not significantly different. This result indicates good reproducibility (mean intensity of 187 ± 18; N = 16) of paper-based indirect ELISA through contact printing using a paper-based stamp (Supplementary Figure 4D).

Supplementary Figure 4. Paper-based indirect ELISA using a combination of wax printing and a paper-based stamp. A) Schematic of the process used in paper-based indirect ELISA. B) Photograph of a paper-based stamp, and paper patterned using wax printing to create test zones. C) Colorimetric results of two sequential printings (i & ii); sixteen test zones (6.7 nM rabbit IgG) were obtained by a single printing step. Two sequential printings show no significant different. D) Measuring color in the test zones using ImageJ[11] allowed an estimation of the mean intensity of color. The intensities in these test zones, obtained from both printing steps, have no significant difference (N = 16). These results indicated good reproducibility for high throughput paper-based indirect ELISA.

Supplementary Figure 4.

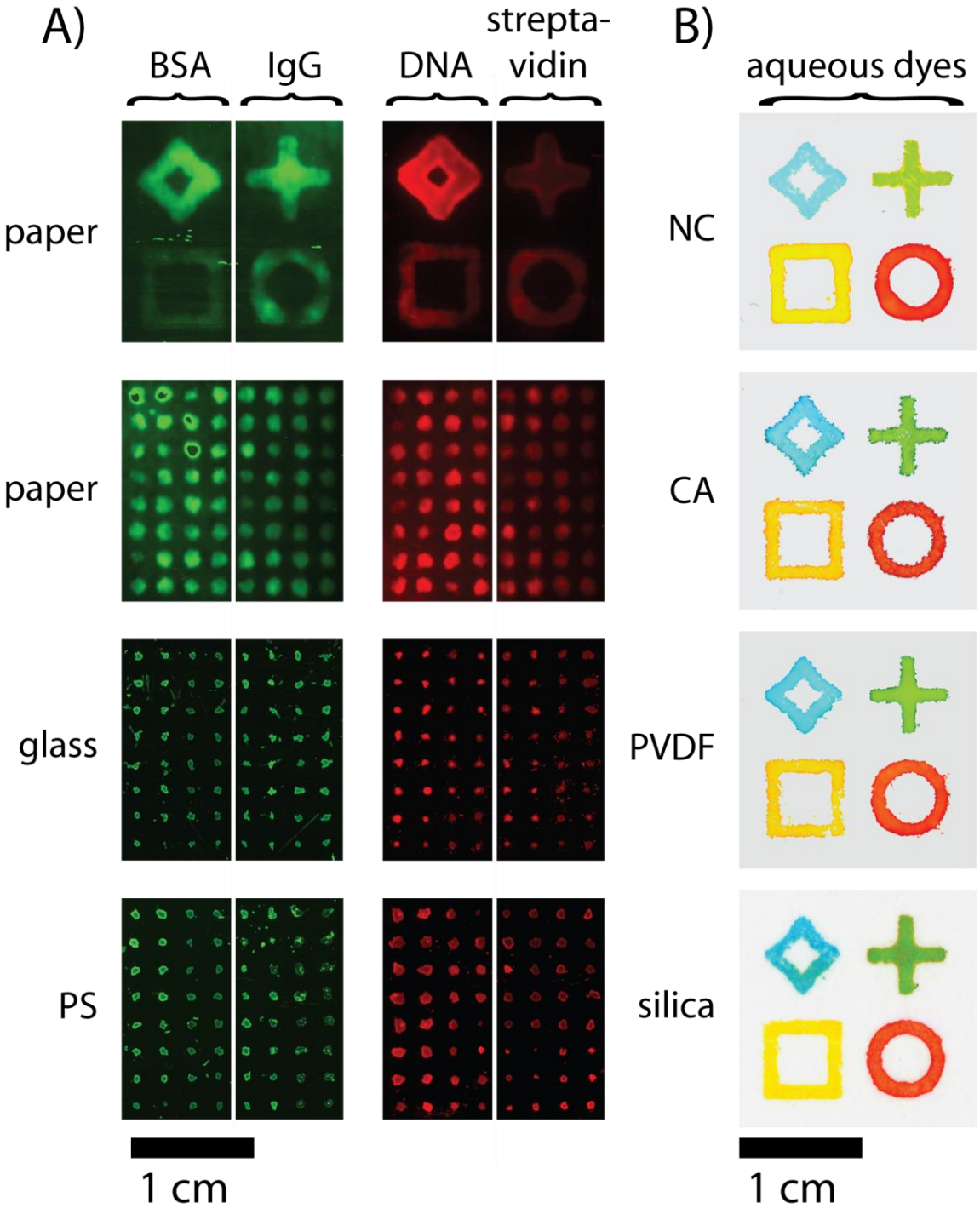


Contact Printing of Different Inks on a Variety of Substrates

To demonstrate that this printing method could be used for other applications (e.g., in bioassays), we printed solutions of fluorescently labeled bovine serum albumin (FITC[12]-BSA), fluorescently labeled rabbit IgG (FITC-IgG), fluorescently labeled streptavidin (Cy3-streptavidin), and fluorescently labeled DNA (Cy3-DNA) on paper, glass and polystyrene (Supplementary Figure 5A). From the images shown in Supplementary Figure 5A—acquired with a fluorescent scanner—we determined that the printed spots on paper were larger than those on glass and polystyrene. This systematic difference is probably due to differences in the volume of solution transferred to the different surfaces, which in turn is related to the differences in porosity, hydrophilicity, and wettability between the substrates. When the fluid contacts the porous paper, capillary action within the paper can cause the fluid to spread laterally. In addition, the nonporous substrates of glass and polystyrene do not facilitate lateral wicking after the stamp is separated from the substrate.

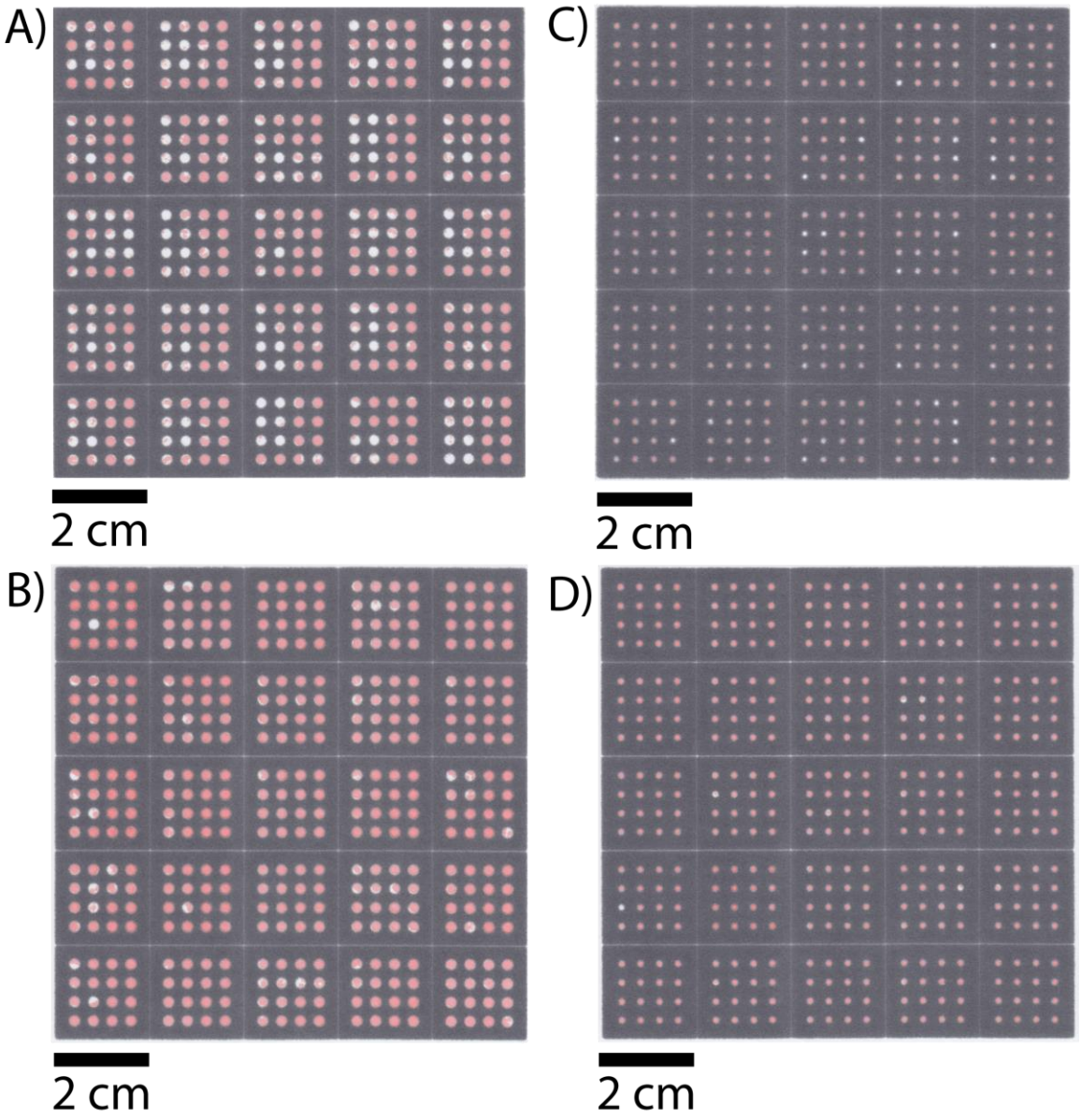
Supplementary Figure 5. Printing a variety of inks on different substrates. A) Patterns of FITC-BSA, FITC-IgG, Cy3-DNA and Cy3-streptavidin printed on paper, glass and non-oxidized polystyrene. The patterns were imaged using a fluorescent scanner (Typhoon imager), and the red and green colors were assigned arbitrarily using Imagequant. B) Patterns of 1-mM Erioglaucine (blue), 12.5-mM Allura red (red), 25-mM Tartrazine (yellow), and 0.5-mM Erioglaucine and 12.5-mM Tartrazine (green), printed on nitrocellulose (NC), cellulose acetate (CA), non-oxidized polyvinylidene fluoride (PVDF), and silica gel. The patterns were imaged using a flatbed fluorescent scanner.

Supplementary Figure 5.



Supplementary Figure 6. Contact printing on pre-patterned Whatman Chromatography paper No. 1. Printing started in the upper-left portion of the shown images, progressed downward, returned to the top of the sheet when reaching the bottom of each column, and stepped to the right. A) Printed spots with a contact time of 2 s. The measured mean diameter was 1.7 ± 0.4 mm with N=358 (coefficient of variation of 24%). B) Printed spots with a contact time of 5 s. The measured mean diameter was 2.1 ± 0.2 mm with N=403 (coefficient of variation of 10%). C) Printed spots with a contact time of 2 s. The measured mean diameter was 0.8 ± 0.1 mm with N=380 (coefficient of variation of 13%). D) Printed spots with a contact time of 3 s. The measured mean diameter was 1.1 ± 0.1 mm with N=380 (coefficient of variation of 9%).

Supplementary Figure 6.



To produce the paper-based stamps and the pre-patterned paper, we used a Xerox printer and a hot plate to create the hydrophilic-hydrophobic boundaries on the individual layers[7]. The pre-patterned substrate and the bottom layer of the stamp making contact with the substrate had circular, hydrophilic regions with nominal diameters of 2 mm and 3 mm. After reflow of the solid ink, the diameters of the hydrophilic regions on both the bottom layer of the stamp and the pre-patterned substrates shrunk to approximately 1 mm and 2 mm, respectively. Supplementary Figure 6 also shows a visible difference in the average diameter of the hydrophilic regions in the pre-patterned substrate. This difference suggests variation in the diameter of hydrophilic regions can result from variability in the reflow of heated wax.

References

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8 Blocking buffer: 0.05% (w/v) Tween-20 and 1% (w/v) bovine serum albumin in PBS.

9 Enzyme-linked antibody: alkaline phosphatase-conjugated anti-rabbit IgG produced in goat (3 μL of a 1:1000 dilution the of stock antibody solution in 0.05% Tween-20 in PBS).

10 Alkaline phosphatase substrate solution: 3 μL of 2.68 mM BCIP, 1.8 mM NBT, 5 mM $MgCl_2$, 100 mM NaCl, 0.05% Tween in 100mM Tris buffer, pH 9.5.

11 Public software from National Institutes of Health: http://rsbweb.nih.gov/ij/

12 FITC: fluorescein isothiocyanate