Electronic Supplementary Material (ESI) for Lab on a Chip

Supplementary Information

Polymerization-based amplification under ambient conditions with thirty-five second reaction times

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Materials and Methods

Materials

Poly(ethylene glycol) diacrylate (M_n=575), triethanolamine, 1-vinyl-2-pyrrolidinone, eosin Y disodium salt, 10x phosphate buffered saline, Triton® X-100, and Tween® 20 were purchased from Sigma Aldrich and used without further purification. Eosin 5-isothiocyanate was obtained from Marker Gene Technology. Streptavidin was purchased from Rockland Immunochemicals Inc. 10x bovine serum albumin blocker solution and EZ-Link Sulfo-NHS-LC biotin were purchased from Pierce/Thermo Scientific. 100x Denhardt's solution was obtained from Bioexpress. Biochip test surfaces containing biotin-functionalized DNA were purchased from InDevR, Inc. Cy3 NHS ester was purchased from Lumiprobe. UltraCruz™ Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology, Inc. Dulbecco's Modified Eagle's Medium was purchased from Lonza Group, Ltd. Fetal bovine serum was purchased from ATCC and penicillin/streptomycin was purchased from EMD Millipore. A BCA Protein Assay Kit with bovine serum albumin standards was obtained from Pierce/Thermo Scientific.

Preparation of Detection Reagents

Eosin initiators were coupled to binding events by reaction of eosin 5-isothiocyanate with a fraction of the solvent-accessible lysine residues of streptavidin as previously described.[6] Stock solutions of free, unconjugated eosin-Y solubilized at a concentration of 5 µM in 1% methanol in distilled water were used to facilitate preparation of three monomer solutions containing final eosin concentrations of 0.3 µM, 0.5 µM, and 0.7 µM. The concentrations of PEGDA, VP, and TEA in these monomer solutions were, respectively, 200 mM, 100 mM, and 150 mM with distilled water as a solvent. PEGDA (M_n=575) as purchased contains 400-600 ppm MEHQ as an inhibitor, and additional purification steps to remove this inhibitor proved unnecessary for polymerization. TEA was used as an alternative to MDEA for its improved water solubility.

Quantification of test surfaces

Calibration Chips (AP-5006, InDevR, Inc.) consisting of 5' amino-50 mer ssDNA-TEG biotin 3' (Spotting Control Oligo, MI-5008, InDevR, Inc.) spotted onto aldehyde-functionalized glass at various dilutions[23] were used as test surfaces. We quantified the number of binding-accessible ssDNA-biotin per square micron in each surface feature using a Cy3-streptavidin conjugate and fluorescence analysis (Agilent microarray scanner) against a Cy3 calibration array (Full Moon Biosystems). Prior to use, surfaces were rinsed with distilled water to remove residual salts and unreacted oligos. The test surface was contacted with 7.5 µg/ml Cy3-streptavidin in 0.75% BSA in 1.5x PBS, 5x Denhardts for five minutes in a humid chamber. This concentration is within the previously determined range of concentrations (1-10 ug/mL) that ensure signals dominated by specific rather than nonspecific binding.[6,10] Sequential rinses with PBST (1x PBS, 0.1% Tween 20), 1x PBS, and ddH_2O were used to remove unbound Cy3-streptavidin. Background-corrected fluorescence signals were compared with a standard curve generated using the Full Moon Biosystems calibration array where features containing only spotting buffer were used to calculate background signal.

Detection of molecular recognition using PBA under ambient conditions

For polymerization studies, test surfaces were contacted with 40 µL of 7.5 µg/ml eosin-streptavidin in 0.75% BSA, 1.5x PBS and 5x Denhardt's Solution for 5 minutes in a humid chamber. To remove unbound initiator, surfaces were rinsed with PBST (1x PBS, 0.1% Tween 20), 1x PBS, and distilled water and wicked dry. Once dry, 40 µL of the prepared monomer solution was contacted with the surface and the surface was irradiated with 522 nm light (30 mW/cm^2, measured using a SPER Scientific Light Meter) from an array of LEDs housed within the ampliPHOX® Reader (InDevR). Unreacted monomer solution was removed by rinsing the surface with distilled water. Remaining hydrogel surface features, if present, were stained with 25 mM eosin (50% methanol, 50% distilled water) for 2 minutes and subsequently rinsed with water to allow visualization of the polymer on the slide surface. Each condition

was repeated a minimum of 5 times. To verify the specificity of binding, competitive binding assays using eosin-streptavidin conjugates that were pre-incubated with an excess of free biotin were performed. To pre-block the eosin-streptavidin conjugates with free biotin, biotin dissolved in DMSO (22 mM) was added to the macrophotoinitiator solution to achieve a final concentration of 550 µM, a >1000-fold excess relative to streptavidin. The final DMSO concentration of the solution incubated with biochip surfaces was 2.5% on a volume basis, and biochips were developed as described above.

Assessing tolerance for complex analyte solutions

A431 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were lysed with Triton X-100 using standard protocols.[24] In brief, A431 cells were grown to confluence on a 100 mm culture plate. The culture medium was discarded and the cells were washed twice with 1xPBS prior to the addition of 1 mL chilled lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4). The cells were incubated on ice with occasional rocking for 25 minutes. The lysate was transferred to a microcentrifuge tube and centrifuged at 18,000xg for 10 minutes at 4˚C. The supernatant was then removed and the total protein concentration, quantified with a BCA assay, was determined to be 0.9 mg/ml. The lysate was diluted to 0.5 mg of mammalian protein/mL with BSA in 1x PBS (for a final concentration of 0.75% BSA in 0.5x PBS) and macrophotoinitiator was added to a final concentration of 375 nM (or 7.5 µg/mL streptavidin). The surfaces were contacted with the macrophotoinitiator in lysate for 5 minutes and developed normally. Macrophotoinitiator was also added directly to undiluted cell lysate (0.9 mg of mammalian protein/mL) for comparison.

Analysis

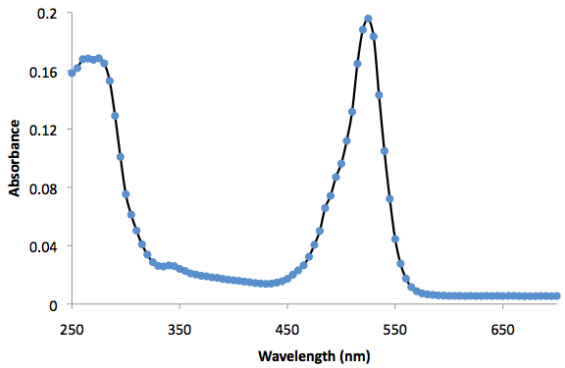
Each surface was imaged using the digital camera built into the ampliPHOX® Reader (InDevR, Inc.) imaging bay. Mean intensity and standard deviation values were calculated for every surface feature and for the background in an automated fashion using software that accompanies the ampliPHOX® Reader. Hydrogel thickness profiles were obtained using a Dektak Stylus Surface Profiler with a 2.5 µm stylus applying a force of 98 µN. Hydrogels were measured in the dry state.

Supplementary Figure 1. Details of EITC-streptavidin coupling, purification, and characterization

Streptavidin (1 mg) was dissolved in 100 uL of 0.1 M sodium bicarbonate buffer, pH 9. Eosin 5-isothiocyanate (EITC, 1 mg) was dissolved in 100 uL of DMSO. 10 uL of the EITC solution was added to the streptavidin solution (100 uL) for a total reaction volume of 110 uL. After gentle pipetting to mix, the reaction was protected from light and maintained at 4˚C overnight. Remaining 10 uL aliquots of EITC in DMSO were stored at -80˚C for future use.

Following overnight reaction, pre-packed Sephadex gel filtration columns for use in microcentrifuges were used to separate unreacted EITC from SA-EITC conjugates. Performing a buffer exchange of the column material into 0.1 M sodium bicarbonate buffer was important for obtaining optimal product recovery, and 1/3 of the reaction mixture was loaded onto each of three Sephadex spin columns. Three columns were used at this scale to avoid overloading the column with uncoupled EITC. The recovered streptavidin-EITC conjugates were pooled and diluted with 890 uL of phosphate buffered saline.

UV-visible absorbance spectroscopy was used to determine the average number of eosin molecules covalently coupled to each protein. Measured absorbance values at 280 nm and 525 nm and measured extinction coefficients in the stated buffer system were used in the following equation to determine an average number of EITC per protein:
$n_{EITC}/n_{SA} = (Abs_{EITC,525}/ \varepsilon_{EITC,525})/[(Abs_{SA,280}-Abs_{EITC,280})/\varepsilon_{SA,280}]$, and $\varepsilon_{EITC,525}$ = 90,200 $M^{-1}cm^{-1}$, $\varepsilon_{EITC,280}$ = 26,800 $M^{-1}cm^{-1}$, $\varepsilon_{SA,280}$ = 173,000 $M^{-1}cm^{-1}$.

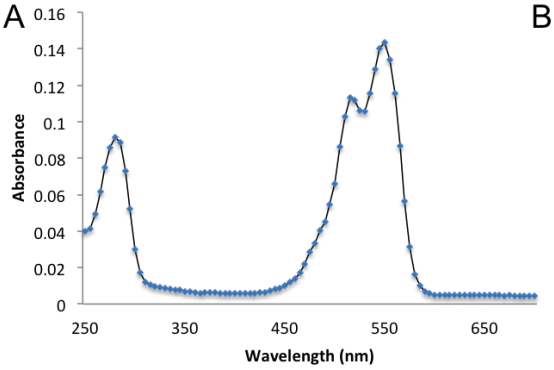


Following characterization, the streptavidin-EITC solution was diluted (1:1 on a volume basis) with glycerol and stored in aliquots at -20˚C for a period of up to six months without an observed loss of binding activity when used as specified in the text of this paper.

Supplementary Figure 2. The number of binding-accessible ssDNA-biotin in each set of features of the test surface was quantified using a Cy3-Streptavidin conjugate, a scanner calibration array and fluorescence analysis

(A) A streptavidin-Cy3 conjugate with a known average number of Cy3 molecules per streptavidin was prepared using the same method outlined above, though with a NHS ester-functional dye in the place of an isothiocyanate dye. An average of two Cy3 were coupled to each protein according to the provided spectral data and the following extinction coefficients: $\varepsilon_{cy3,552}$ = 150,000 $M^{-1}cm^{-1}$, $\varepsilon_{cy3,280}$ = 12,000 $M^{-1}cm^{-1}$ $\varepsilon_{SA,280}$ = 173,000 $M^{-1}cm^{-1}$. (B) A biochip test surface was reacted with the Cy3-streptavidin conjugate as described in the text and imaged. The average signal to noise ratios (C) were compared with a standard curve generated using a Full Moon Biosystems calibration array (D). The signal intensities on the surface were determined using Metamorph Basic to compute average intensities within specified regions of constant area. The signal to noise ratio is defined as the difference between the signal and the background (in this case, the average signal of the three buffer spots) divided by the standard deviation of the background ———————— . The fluorescence images were obtained using an Agilent microarray scanner with an excitation wavelength of 532 nm (20 mW) and emission wavelengths between 550 nm and 610 nm. The intensity scale is 0 to 10000 and the PMT setting was 10%.

A



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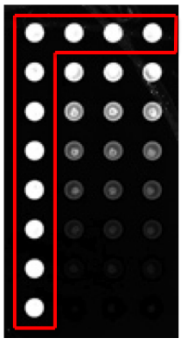
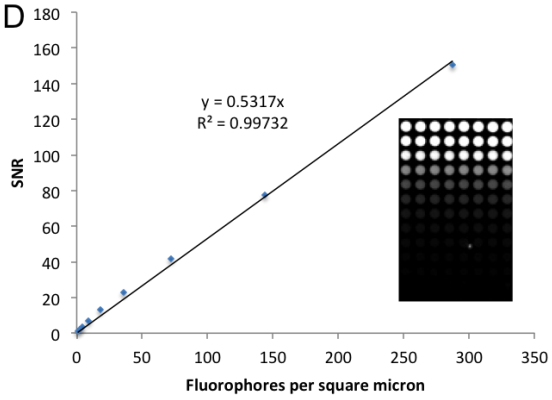


C

Position	SNR	#Biotin/µm²
0	113	110
1	103	100
2	54	50
3	29	27
4	16	15
5	7	6
6	2	
7	1	

D



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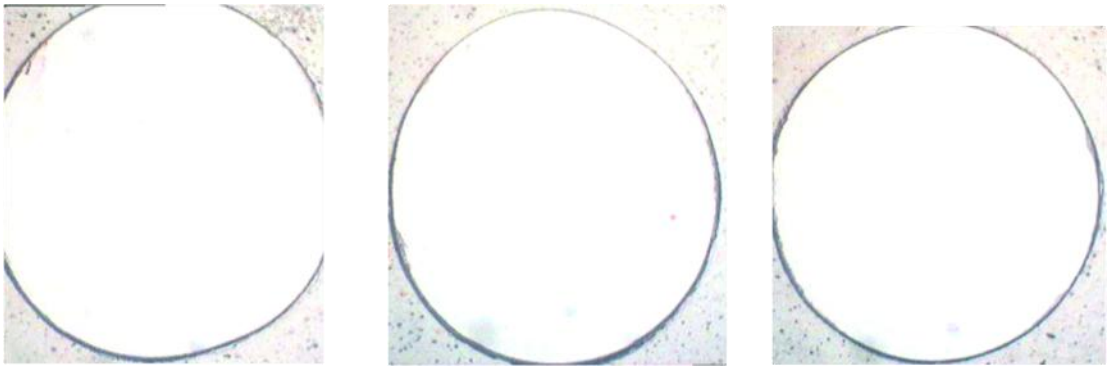
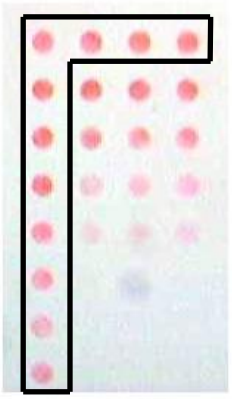
Supplementary Figure 3. Competitive binding assays using eosin-streptavidin pre-incubated with a 70-fold excess of free biotin confirmed the specificity of the binding interaction. Displayed are the negative controls for monomer solutions containing 0.3 µM, 0.5 µM, and 0.7 µM eosin, respectively.



Supplementary Figure 4. Colorimetric detection of molecular recognition using PBA under ambient conditions. The monomer solution contained a bulk concentration of 0.3 μM eosin and the irradiation time was 100 seconds with 522 nm light from LEDs (30 mW/cm^2). This table includes average thickness data as determined using profilometry.



Position	Biotin/μ m^2	Average Intensity	Average Thickness
0	110	66.7 ± 11.6	597.6 ±267.5
1	100	73.3 ± 5.7	1007.7 ± 68.5
2	50	61.7 ± 6.0	531.9 ± 73.5
3	27	41.7 ± 2.9	230.4 ± 30.2
4	15	35.8 ± 2.0	117.8 ± 9.2
5	6	25.8 ± 1.2	
6		23.6 ± 1.0	
7	0 (spotting buffer)	25 ± 1.1	
Background	N/A	26 ±1.8	

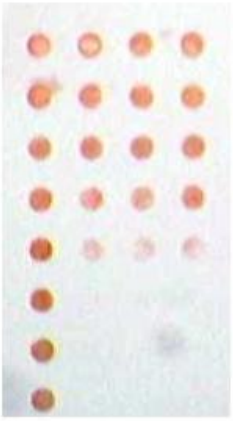
Table 1. Average Film Thickness

Position	Biotin/µm^2	Film Thickness (nm) (0.3 µM Eosin)	Film Thickness (nm) (0.5 µM Eosin)	Film Thickness (nm) (0.7 µM Eosin)
0	110	747.3 ± 211.6	868.5 ± 226.2	560.6 ± 165.5
1	100	844.7 ± 230.5	707.5 ± 55.3	546.1 ± 344.5
2	50	468.0 ± 90.3	744.0 ± 353.3	466.5 ± 47.4
3	27	271.3 ± 57.8	389.6 ± 246.6	401.8 ± 130.5
4	15	238.1 ± 170.1	334.3 ± 42.1	336.8 ±91.8
5	6	327.1 ± 18.4	533.0 ± 334.9	440.7 ± 249.5

Table 2. Average Pixel Intensity

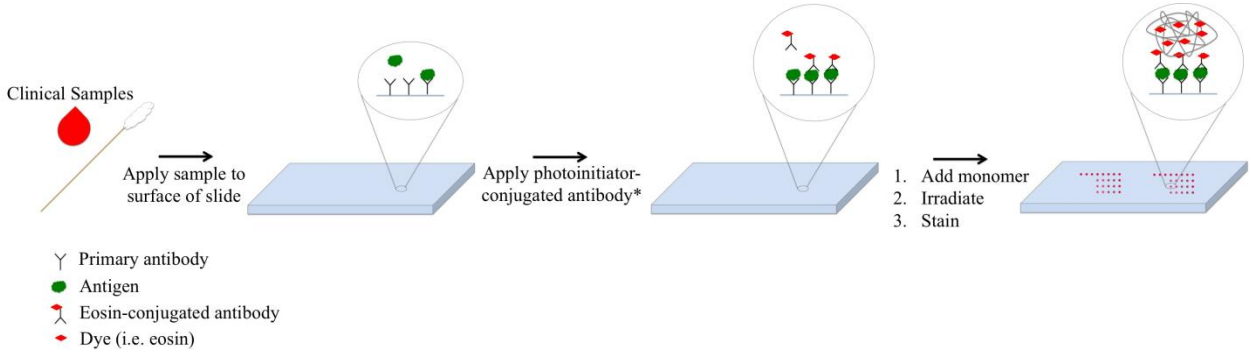
Position	Biotin/µm^2	Average Pixel Intensity (0.3 µM Eosin)	Average Pixel Intensity (0.5 µM Eosin)	Average Pixel Intensity (0.7 µM Eosin)
0	110	63.5 ± 6.0	49.5 ± 5.5	56.1 ± 3.5
1	100	67.1 ± 9.0	47.5 ± 7.6	54.8 ± 3.1
2	50	59.1 ± 10.4	51.3 ± 10.3	49.8 ± 5.1
3	27	42.7 ± 5.0	44.8 ± 3.2	46.8 ± 6.0
4	15	39.6 ± 6.6	41.9 ± 7.8	38.6 ± 6.6
5	6	30.2 ± 10.6	44.6 ± 10.3	37.0 ± 14.5
6		24.4 ± 1.0	25.0 ± 0.7	25.4 ± 1.3
7	0 (spotting buffer)	24.4 ± 0.8	25.0 ± 0.8	24.6 ± 0.9
Background	N/A	25.6 ± 0.3	25.4 ± 0.3	25.5 ± 0.3

Supplementary Figure 5. Providing the macrophotoinitiator in a mammalian cell lysate solution rather than in buffer has a negligible effect on the system sensitivity. The surface was contacted with macrophotoinitiator in A431 cell lysate diluted to 0.5 mg of mammalian protein/mL with BSA in PBS. The surface was then developed using an aqueous monomer solution containing 0.5 µM eosin and irradiated for 70 seconds. An identical sensitivity of ≥ 15 possible molecular recognition events per square micron was observed without any increase in background (non-specific signal). For macrophotoinitiator spiked directly into undiluted lysate with no added BSA, sensitivity decreased to ≥ 27 possible molecular recognition events per square micron without an increase in background signal.



Position	Biotin/μm^2	Average Intensity
0	110	69.6 ± 9.0
1	100	69.1 ± 3.6
2	50	67.6 ± 4.7
3	27	61.4 ± 8.7
4	15	38.5 ± 4.7
5	6	25.8 ± 1.2
6		25.0 ± 1.3
7	0 (spotting buffer)	24.6 ± 1.0
Background	N/A	25.0 ± 1.7

Supplementary Figure 6. For application of this method in real-world assays, binding events in addition to the recognition of biotin by streptavidin will be required. These additional binding events and the steps that will be added to the assay are represented below. Present work is focused on application of the air-tolerant amplification chemistry reported here in assays of clinical samples that do not require pre-purification or concentration steps. In previous inert-gas purged PBA, influenza subtyping from crude lysates and the detection of nucleic acids in serum (single nucleotide polymorphisms) have been demonstrated (refs. 14 and 12 in the text, respectively).



* A biotin-labeled antibody in combination with the streptavidin-$(eosin)_3$ conjugate used in this work may be substituted for an initiator-labeled antibody, and alternatives to antibodies are also suitable.