Electronic Supporting Information

An automated integrated platform for rapid and sensitive multiplexed protein profiling using human saliva samples

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Materials

Poly(ethylene glycol) with an average molecular weight of 200 (PEG 200), polyvinylpyrrolidone with an average molecular weight of 10,000 (PVP), 10× phosphate buffered saline (PBS), and tris buffered saline with 0.05% Tween 20 (TBS/Tween 20) were purchased from Sigma-Aldrich (St. Louis, MO). SEA Block Blocking Buffer, SuperBlock Blocking Buffer, Protein-Free Blocking Buffer, Blocker Bovine Serum Albumin (10% BSA) in PBS, PBS Starting Block Blocking Buffer, and TBS Starting Block Blocking Buffer were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Poly(ethylene glycol) with an average molecular weight of 10,000 (PEG 10k) was purchased from Alfa Aesar (Ward Hill, MA). 2-(methoxy(polyethyl(ethoxy)propyl)trimethoxy)silane, 90% (PEG-silane moiety) and N-(triethoxysilylpropyl)-o-polyetheylene oxide urethane (PEG terminated urethane moiety) were purchased from Gelest (Morrisville, PA). Streptavidin-Alexa Fluor 488 (AF488) conjugate was purchased from Invitrogen (Carlsbad, CA).

Surface passivation protocols:

Initial efforts focused on static passivation agents. Silanization of PDMS and glass surfaces to reduce protein adhesion has been well documented. Poly E-323 is a cationic polymer that has also shown promise in reducing protein and peptide adhesion in glass capillaries and microchips, as well as PDMS molded electrospray tips.[1-3] For these reasons, two silanes, a PEG terminated urethane moiety and a PEG-silane moiety known to reduce protein adhesion, and Poly E-323 were chosen as passivation agents to test. PDMS microchannels were filled with either neat PEG terminated urethane moiety, PEG-silane moiety, or 6% Poly E-323 (pH 7, synthesized in-house and diluted in deionized water) immediately after oxygen plasma treatment and allowed to incubate at room temperature for 10 min before rinsing with deionized water followed by PBS with 0.1% BSA. VEGF sandwich immunoassays were performed at room temperature by incubating VEGF for 1 h, biotinylated detection antibody for 30 min, and streptavidin-AF488 for 20 min before rinsing with TBS w/0.05% Tween 20 for 10 min. Fluorescence background intensities from images of the PDMS surface of each microchip were then compared.

Dynamic surface coatings are buffer additives that reduce protein adhesion. It is thought that preferential adsorption of the buffer additives prevents protein adhesion to the surface.[4] Several commercially available blocking buffers were evaluated, as well as PBS solutions containing 0.1% BSA, 10% BSA, 0.1% PEG 10k, 0.1% PEG 200, and 0.1% PVP. Separate microchips were passivated with each solution for 1 h at 4 °C before performing immunoassays using the same procedures that were used for the static passivation studies.

Surface passivation results

Razunguzwa et. al. previously demonstrated reduced protein adhesion in glass capillaries using the PEG-terminated urethane moiety;[5] however, we observed that this coating did not adequately reduce adsorption of the streptavidin-AF488 complex to the PDMS surface, as the background signal intensity for microchips passivated with this coating was at least 60% higher than for the other surface coatings tested. Poly E-323 proved more effective at reducing the dye adhesion; however, this molecule bonds to the surface via an electrostatic interaction that weakens over time, perhaps necessitating regeneration of the surface coating. The PEG-silane moiety proved to be the most effective static coating tested, giving a background intensity that was nearly 50% lower than the PEG-urethane moiety and 15% lower than the Poly E-323 coating. However, the time required to prepare a chip using this high viscosity reagent resulted in long preparation times. As ease of fabrication is a concern, further investigations focused on dynamic surface coatings that required less preparation time.

The average signal intensity and S/N values for each dynamic coating are shown in Table S1. A marked increase in the average signal intensity was found when 0.1% PEG 10k was added to PBS. While the SeaBlock buffer and 0.1% PVP in PBS gave comparable signal intensities, the background and noise values for these buffers were much greater, resulting in a lower overall S/N value. For these reasons, 0.1% PEG 10k in PBS was chosen as the passivation agent for all future immunoassays.

Table S1. Results of dynamic coating studies.

Dynamic Additive	VEGF Intensity (a.u.)	Background Intensity (a.u.)	Noise (a.u.)	S/N
0.1% PEG-10k*	39100	4200	35	1117
SeaBlock blocking buffer	33300	6000	56	600
0.1% PVP*	28000	4500	56	500
0.1% BSA*	22300	4800	48	465
Protein-free blocking buffer	23500	6100	56	420
0.1% PEG 200*	12700	4100	36	350
10% BSA*	17400	6100	50	348
SuperBlock blocking buffer	9800	4500	46	213

*passivation additive was added to PBS to give the final assay buffer

Microfluidic chip loading device

A microsphere loading device (shown in Fig. S1) was designed using BobCAD-CAM software (BobCAD-CAM, Clearwater, FL) to automate and standardize the microsphere loading process. The body of the loading device was machined from polycarbonate (McMaster Carr, Chicago, IL) using a MicroMill DSLS 3000 (MicroProto Systems, Chandler, AZ) computer numeric controlled machine. A solenoid actuator (part number 6873K21, McMASTER-CARR, Robbinsville, NJ) was affixed to the top plate of the loader so that its rod would be aligned with the array when the chip was inserted into the loader. Actuation of the solenoid was controlled by a switch and 12 VDC power supply. After the channel was filled with microsphere slurry, the chip was inserted into the loading device, and the solenoid was actuated 60−80 times within a 3 min timeframe so that it pressed the PDMS against the glass and trapped microspheres in the microwells. The channel was then flushed with buffer to remove loose microspheres.

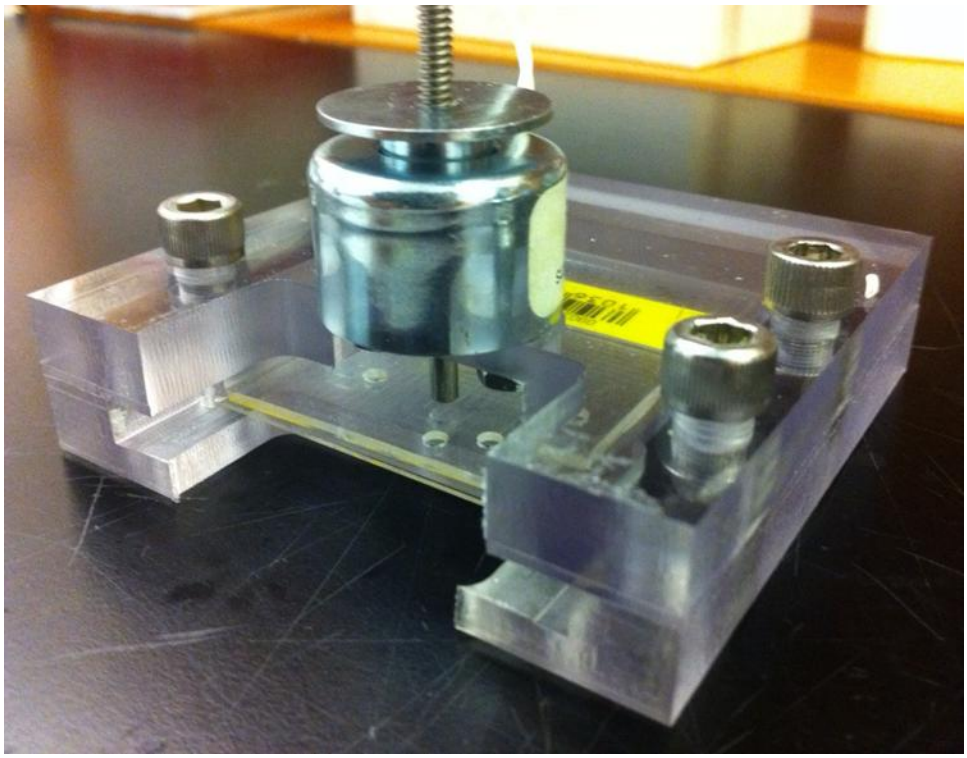

Fig. S1 Image of the microfluidic chip loading device.

System characterization

The platform was characterized using recombinant protein standards at different concentrations. Three independent measurements were performed at each concentration and the average responses were curve fitted using a 4-parameter logistic regression (4PL) model, shown in Equation S1[6]:

$$Y = D + \frac{(A - D)}{1 + \left(\frac{x}{C}\right)^{B}} \qquad \text{(Equation S1)}$$

Y is the fluorescence response on the SDReader, D is the estimated response at infinite concentration of the analyte, A is the estimated response at zero concentration of the analyte, x is the concentration of the analyte, B is the slope of the curve at the inflection point, and C is the midrange concentration. Data were analyzed using Origin (OriginLab Corporation, Northampton, MA).

Detailed saliva analysis process

The interface of the SDReader control window is shown in Fig. S2. After the barcodes of the saliva sample and microfluidic chip were typed or scanned and the fully assembled chip was fed into the SDReader, the following automated analysis procedure was initiated by pressing the "Run Assay" button. Valves 2-4 were closed while valve 1 was opened, the vacuum pump was turned on, and the saliva sample in Input A (Fig. 2a) was introduced into the microsphere array by the nagetive vacuum force incubated for 30 min with ultra-flow (in which the reagents were flowed for 5 s every 2 min to introduce fresh reagents into the array). After the sample incubation, valve 1 was closed and valve 4 was opened, the pump was turned on, and the microsphere array was washed by washing buffer from Input D (Fig. 2a) for 2 min. Then, valve 4 was closed and valve 2 was opened, the pump was turned on, and the mixture of biotinylated detection antibodies in Input B (Fig. 2a) was introduced and incubated with the microsphere array for 15 min. The washing step was repeated once, and then the microsphere array was incubated with streptavidin-dye from Input C (Fig. 2a) for 8 min. A final wash of 3 min was performed after that to remove all unbound molecules in the microsphere array. The objective lens was then automatically driven by a linear actuator and the optimal focusing position was determined as described below. The microsphere array was subsequently imaged under white and green LED illumination using exposure times of 100 ms and 1 s, respectively. Both images were automatically saved on the hard drive with the saliva sample and microfluidic chip barcodes contained in the file names.

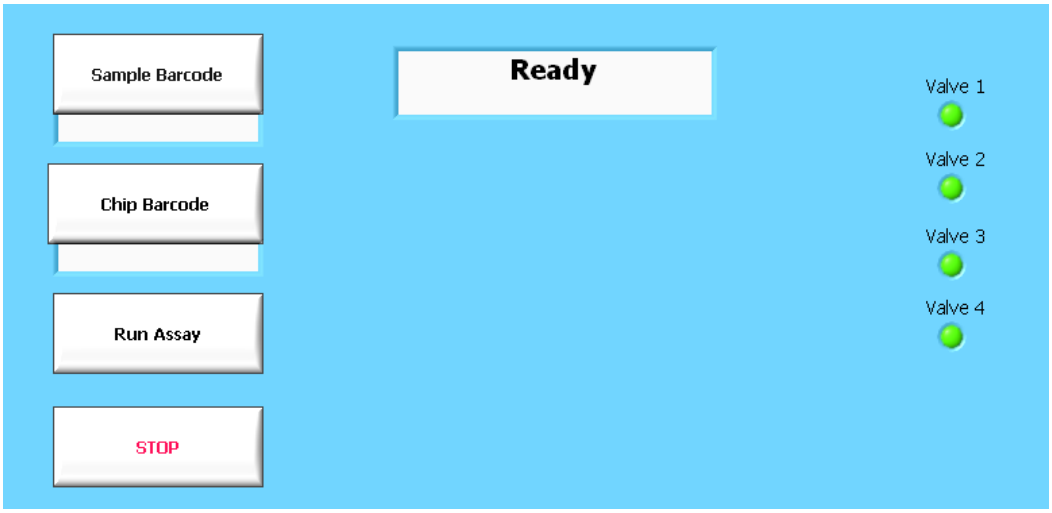

Fig. S2 Interface of the SDReader device.

Microwell geometries for microsphere loading and retention

Thirty-two different microwell geometries were fabricated by DRIE on six-inch silicon wafers that were used to cast PDMS chips to determine the critical dimensions needed for high occupancy microsphere loading. Fig. S3a shows a schematic of the mask geometries used for the microwells etched on silicon wafers to create master molds. Geometries include round, oval, square, and various polygons. Chips cast from these molds were evaluated in triplicate for microsphere loading and retention studies to determine optimum geometries. It was noted that the photolithography resulted in mold geometries that could be reduced in diameter by ≈ 25%. Other geometries such as #10 or #32 did not transfer with high fidelity to the substrate, resulting in large size and shape deviations. Examples of the PDMS microwell features cast from these structures and loaded with 3-µm diameter microspheres are shown in Fig. S3b. Fig. S4 shows a graph of the average number of microspheres loaded into and retained by an array of 160 microsphere wells fabricated from each mask geometry. It was determined that optimal microsphere loading was achieved with mask geometries having at least one dimension slightly smaller than the microsphere diameter (nominally 3.0 µm). For example, with wells 3 µm deep, fabricated from a mask with 2.7 µm diameter circles loaded and retained microspheres better than those fabricated from 3.3 µm circles. Oval geometries and other geometries with at least one dimension greater than 3.75 µm were prone to double loading of microspheres.

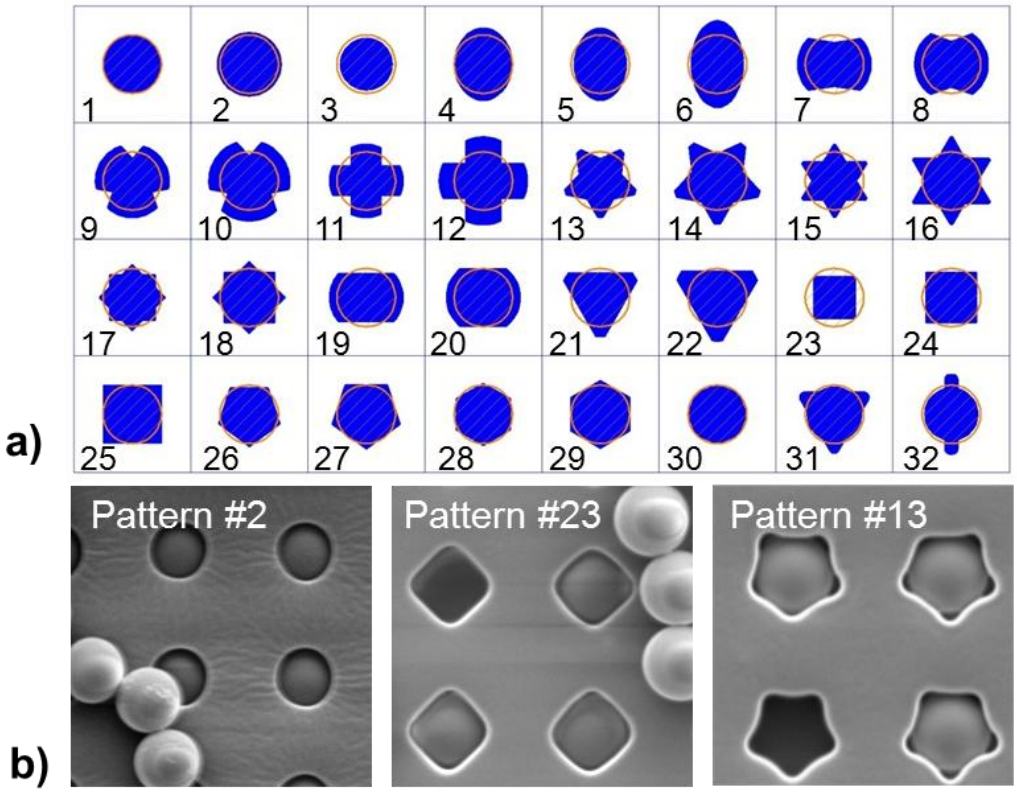

Fig. S3 (a) Schematic of patterns evaluated for microsphere loading efficiency (blue) with overlay showing 3 µm microsphere diameter (orange cricle). (b) Representative SEM images of cast PDMS arrays loaded with 3 µm microspheres.

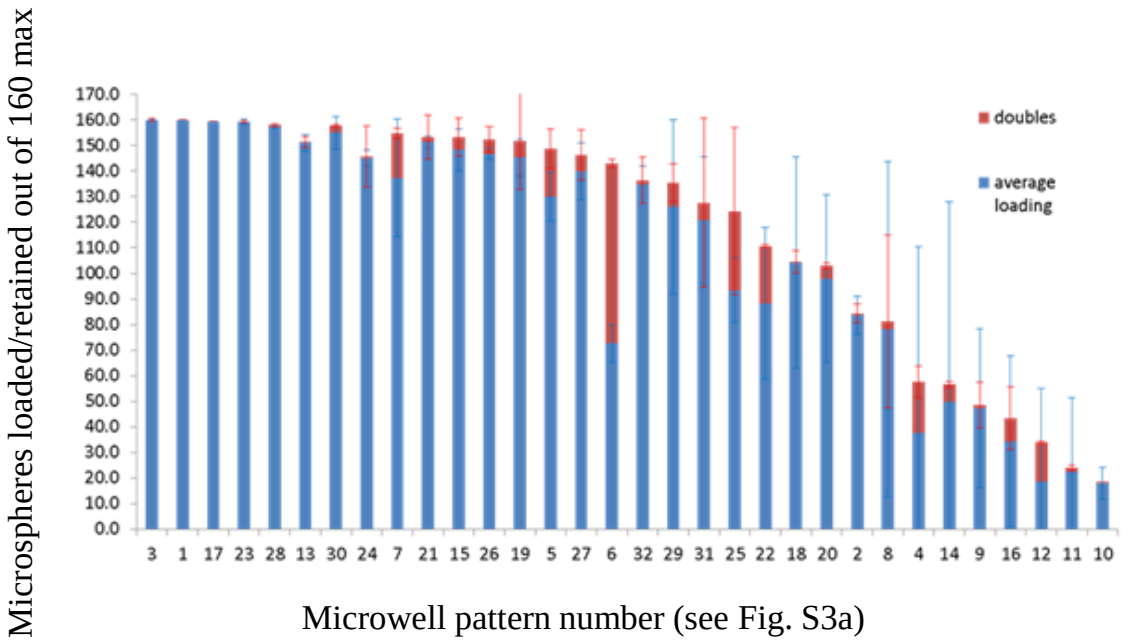

Fig. S4 Average number of microspheres loaded into and retained by an array of 160 microsphere wells fabricated from each mask geometry.

Valve system integration

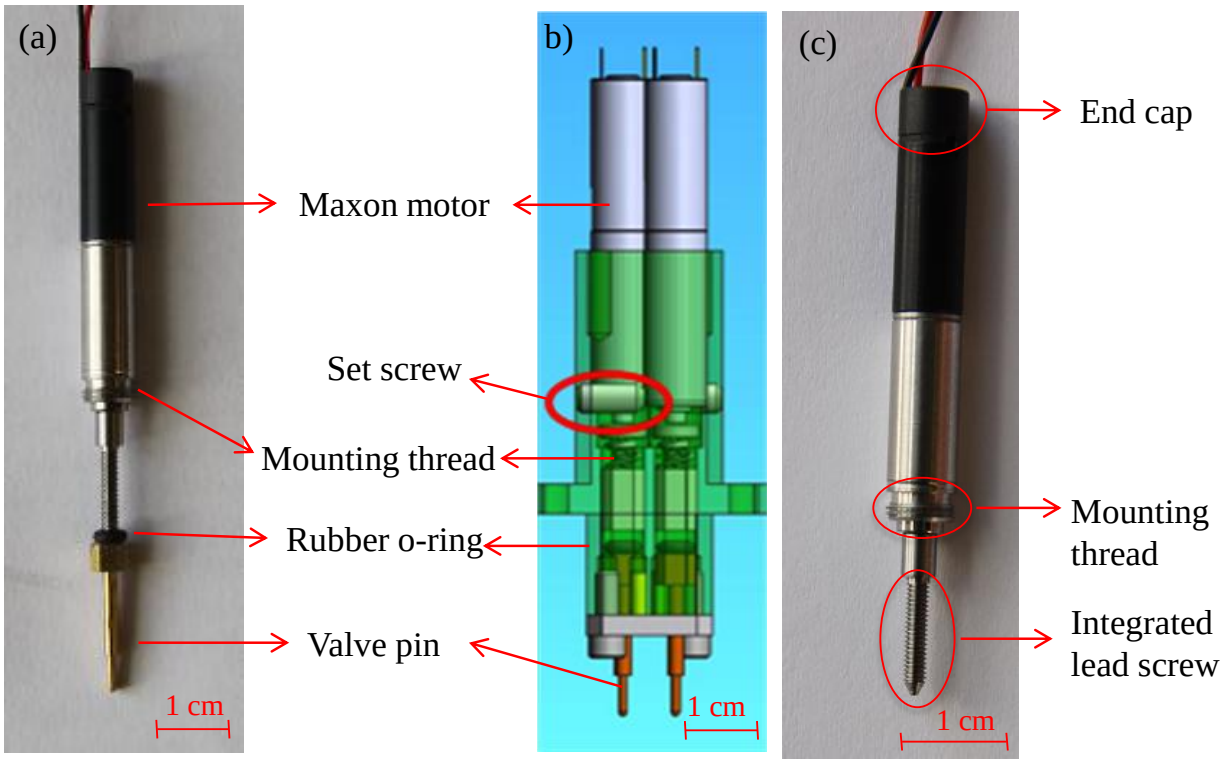

Fig. S5 Design of the actuator valve system on the SDReader. (a) Photograph of an actuator valve, a rubber o-ring, and a valve pin. (b) Cartoon of the actuator valve system assembly. The lead screw is threaded into an actuator mount (shown in green) and the motor is held by the mounting thread and a set screw (shown in the red circle). The pressures generated by the motors are applied on the valve pins (shown in orange), which close the microfluidic channels. The rubber o-ring between the actuator and the pin is used to buffer the force applied on the pin. (c) A photograph of the Maxon motor.

SDReader valve tests

Table S2. Variation of stop currents of the motors among four actuators for 17 cycles.

Actuator	Current at stop (A)		
	Average	SD	Error (3 × SD)
1	0.132	0.002	4.5%
2	0.133	0.003	6.8%
3	0.133	0.003	6.8%
4	0.134	0.003	6.7%
Overall	0.133	0.003	6.8%

Cross-reactivity tests

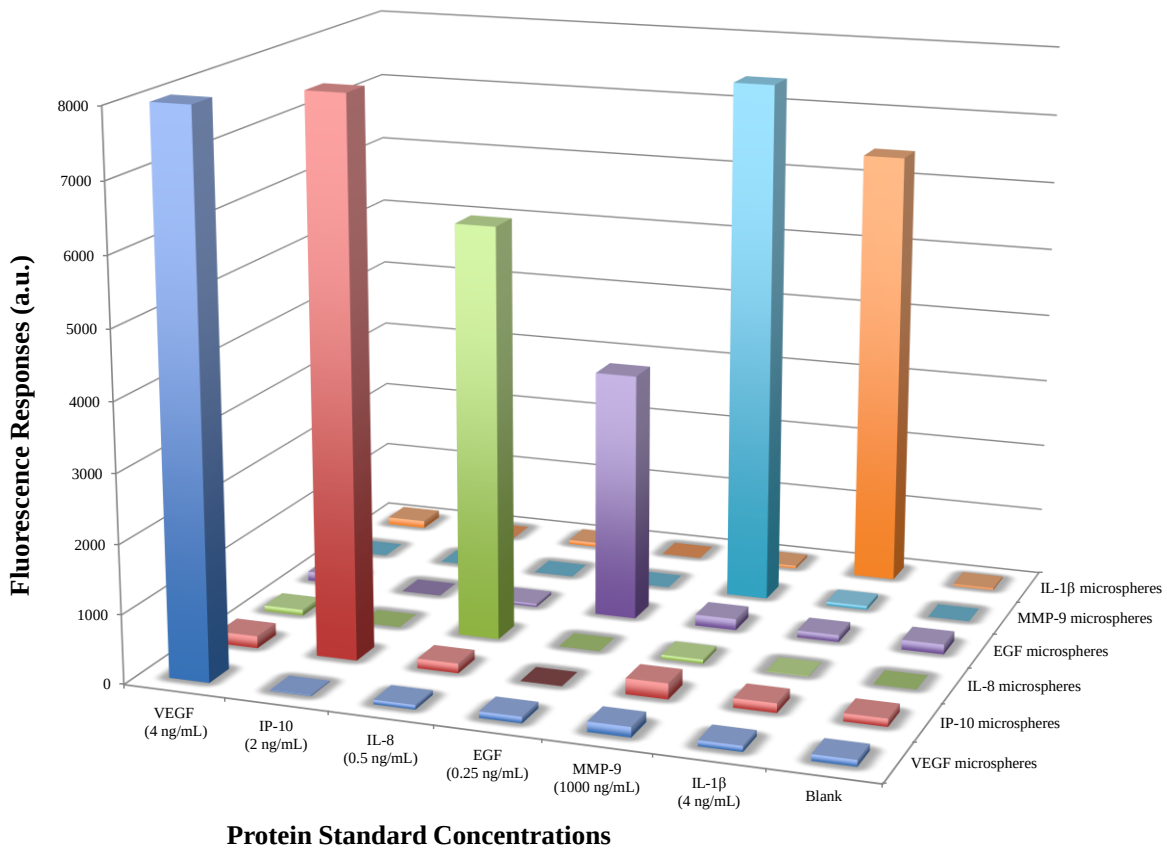

Fig. S6 Results for both the blank and cross-reactivity experiments. Six chips loaded with seven types of microspheres (including one negative control) were sequentially incubated with a single protein, a mixture of all six detection antibodies, and SARPE in each experiment. The signal intensities of different microsphere types (shown in different colors) are shown. The average responses from three blank tests (using PBSS buffer) are listed on the right as a reference.

Concentrations of protein standard mixtures for calibration curves

Table S3. Concentrations of the protein standard mixtures analyzed for the six-plex calibration curves (Fig. 5).

Analytes	1	2	3	4	5	6
VEGF (pg mL^{-1})	8000	3200	1280	512.0	204.8	81.92
IP-10 (pg $mL^{-1)}$	2000	800.0	320.0	128.0	51.20	20.48
IL-8 (pg mL^{-1})	1000	400.0	160.0	64.00	25.60	10.24
EGF (pg mL^{-1})	1000	400.0	160.0	64.00	25.60	10.24
MMP-9 (ng mL^{-1})	500.0	200.0	80.00	32.00	12.80	5.120
IL-1β (pg mL^{-1})	8000	3200	1280	512.0	204.8	81.92

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