# High-throughput multi-antigen microfluidic fluorescence immunoassays

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Here we describe the development of a high-throughput multi-antigen microfluidic fluorescence immunoassay system. A 100-chamber polydimethylsiloxane (PDMS) chip performs up to 5 tests for each of 10 samples. In this particular study system, the specificity of detection was demonstrated, and calibration curves were produced for C-reactive protein (CRP), prostate-specific antigen (PSA), ferritin, and vascular endothelial growth factor (VEGF). The measurements show sensitivity at and below clinically normal levels (with a signal-to-noise ratio >8 at as low as 10 pM antigen concentration). The chip uses 100 nL per sample for all tests. The developed system is an important step toward derivative immunoassay applications in scientific research and "point-of-care" testing in medicine.

#### **INTRODUCTION**

The ongoing revolution in biological sciences has generated high hopes for the advent of true personalized/preventive medicine. While the necessary biological tools are being developed at a fast pace, it has become clear that their cost, operation, and manufacturability are equally challenging issues that must be solved before the new methods can be widely accepted in medical practice. In the particular case of diagnostics, decentralized "near-patient" or "pointof-care" testing (1) has attempted to provide fast quantitative results at the bedside or in the clinic, thereby decreasing hospital stays, eliminating transportation and administrative expenses, and decreasing errors from mishandling and miscommunication. While a few single-analyte systems (1) have been developed (e.g., the now commonplace Glucometer<sup>®</sup>), the enormous potential for decentralized testing remains untapped because the vast majority of medical diagnostics is still conducted in clinical laboratories and with the use of large equipment.

A way for ubiquitous near-patient and point-of-care testing to reach fruition is for the current biological techniques to be reduced from the macroscale to the microscale, in a multi-analyte high-throughput format, preferably on handheld devices. In particular, reducing immunoassays to microfluidic scales has been extensively explored in recent years. Many approaches have been proposed, involving glass (2-8), TiO<sub>2</sub> (8), silicon (9,10), and silicone (9,11-17) devices, but none possesses all of the desirable qualities: (i) capability to measure multiple antigens and samples per device, (ii) industrially feasible fabrication, (iii) parsimony of sample and reagents, (iv) adequate sensitivity and specificity, and (v) good reliability and reproducibility.

Here we report on a high-throughput multi-antigen high-specificity, highsensitivity reproducible polydimethylsiloxane (PDMS) microfluidic system quantifying four representative blood analytes at the clinically relevant levels. An active microfluidic matrix (18) utilizes arrays of integrated micromechanical valves (19) to direct pressure-driven flow and multiplex analyte samples with immunoassay reagents. Enzyme-linked immunosorbent assay (ELISA)-like fluorescence immunostacks are formed in the microchambers at the intersections of sample and reagent channels. The fluorescence signals from these microchambers quantify the captured antigens. The 100-chamber prototype device can conduct 5 tests for each of 10 samples (with 2 replicates per sample-test combination). This test matrix could be expanded to significantly larger numbers (20).

We chose blood analytes for the initial validation of the system because blood tests represent an example of routine use of immunoassays. The current "gold standard" clinical technology is typically based on ELISA and requires 0.5–2 mL of sample per test per patient. High kit and instrumentation costs dictate centralization of measurements to large clinical or reference laboratories, resulting in transportation and batch delays of up to 14 days between the phlebotomist appointment and the final results. Such delays and the macroscale

<sup>1</sup>University of Southern California, Los Angeles, <sup>2</sup>California Institute of Technology, Pasadena, and <sup>3</sup>Stanford University, Stanford, CA, USA of samples and reagents increase costs in today's fast-paced, expensive healthcare environment. By contrast, the system described here uses only 100 nL of sample, while simultaneously measuring C-reactive protein (CRP), prostate-specific antigen (PSA), ferritin, and vascular endothelial growth factor (VEGF) within the clinically significant range. The system also uses only 300 nL of antibodies (as low as 0.8 ng) per assay to measure all 10 samples. Therefore, the microfluidic miniaturization of immunoassays described here may pave the way to efficient and portable handheld devices.

#### MATERIALS AND METHODS

#### Reagents

Chip fabrication. Hexamethyldisilazane (HMDS) adhesion promoter was obtained from ShinEtsuMicroSi (Phoenix, AZ, USA); the photoresist Shipley SJR 5740 from MicroChem (Newton, MA, USA); tetramethylchlorosilane (TMCS) from Sigma (St. Louis, MO, USA); PDMS Sylgard 184 from Dow Corning (Midland, MI, USA); and ArrayIt<sup>®</sup> SuperEpoxide SME slides from TeleChem International (Sunnyvale, CA, USA).

Antibodies and antigens. PSA antigen, monoclonal PSA antibody, ferritin antigen, monoclonal ferritin antibody, and monoclonal CRP antibody were procured from Fitzgerald Industries (Concord, MA, USA); VEGF antigen and antibodies and biotinylated CRP antibody from R&D Systems (Minneapolis, MN, USA); PSA biotinylated antibody from Lab Vision (Fremont, CA, USA); ferritin biotinylated antibody from U.S. Biological (Swampscott, MA, USA); and CRP antigen from EMD Biosciences (Calbiochem<sup>®</sup>; San Diego, CA. USA).



**Figure 1. Microfluidic immunoassays chip.** (A) A 100-chamber  $22 \times 35$  mm PDMS chip bound to an epoxide slide allows 5 tests of each of 10 samples, with 2 chambers per sample-test combination. (B) Functional diagram of the entire chip. Control channels (blue contours) convey pressure to open and close microvalves that direct pressure-driven feeds of reagents along flow channels (pink contours). Each intersection of flow channels in the central test matrix forms a microchamber where an immunostack is constructed. Monoclonal antibodies flowing from inputs D1–5 to exhausts DE1–5 bind to the epoxide coating of the microchannel floor. Buffer flushes from input DB to exhausts DE1–5 and from input SB to exhausts SE1–10 passivate remaining epoxide groups. Samples are fed in parallel from inputs S1–10 to exhausts SE1–10 and pumped along closed circular 10 nL paths through the capture microchambers. Biotinylated polyclonal antibodies fed from inputs A1–5 to exhausts DE1–5 complete the immunostacks in the microchambers. Labeled streptavidin fed from input SA to exhausts SE1–10 binds to the immunostacks. The detected fluorescence signal quantifies the captured antigens. (C) Functional diagram of an individual coliseum. Here control channels and valves are drawn in red and flow channels in blue. Comb-like valve arrays enclose a pair of immunoassay chambers for each of five tests. Valve arrays 1, 2, and 3 pump the sample in a circle along the coliseum [e.g., clockwise for actuation order (1, 2, 3)] with a lap time of 20 s. (D) Typical fluorescence image of a microchamber. A VEGF test of a 0.3 nM sample is shown. PDMS, polydimethylsiloxane; DB, derivatization buffer; SB, samples buffer; SA, streptavidin; VEGF, vascular endothelial growth factor.

Fluorescent probes and buffers. Streptavidin Alexa Fluor<sup>®</sup> 555 was supplied by Invitrogen (Molecular Probes<sup>TM</sup>; Carlsbad, CA, USA). Lyophilized commercial antigens and antibodies were reconstituted in phosphate-buffered saline (PBS)  $1\times$  buffer from Irvine Scientific (Santa Ana, CA, USA), pH 7.5. Bovine serum albumin (BSA) was added to the same to produce the PBS 0.1% BSA buffer. The passivation buffer was 10 mM Tris, 10 mM NaCl, pH 8.0, made from powdered Tris and NaCl (both from Sigma).

#### **Microfluidic Device Fabrication**

Mold fabrication. PDMS microfluidic chips with integrated micromechanical valves were built using soft lithography as previously described (19), with the following modifications. Silicon wafers were exposed to HMDS vapor for 3 min. The wafers were coated with Photoresist SPR 220-7 by spinning at 2000 rpm for 60 s on a WS-400A-GNPP/LITE spincoater (Laurell Technologies, North Wales, PA, USA). The wafers were baked at 105°C for 90 s on a hotplate. UV exposure through black-and-white transparency masks was performed for 1.75 min on a Karl Suss MJB3 mask aligner (Karl Suss America, Waterbury, VT, USA). The molds were then developed for 2 min in 100% MicroChem 319 developer (MicroChem). Flow layer molds were baked at 140°C for 15 min on a hotplate to melt and round the flow channels. Molds were characterized on an Alpha-Step 500 (KLA-Tencor, Mountain View, CA, USA). Channel height was between 9 and 10  $\mu$ m. The control channel profile was rectangular, while the flow channel profile was parabolic. Except for the height measurements, the mold fabrication was conducted in a Class 10,000 clean room.

Chip fabrication. Molds were exposed to TMCS vapor for 3 min. PDMS in 5:1 and 20:1 ratios were mixed and degassed using an HM-501 hybrid mixer and cups from Keyence (Long Beach, CA, USA). Thirty-five grams of the 5:1 were poured onto the control mold in a plastic Petri dish wrapped with aluminum foil. Five grams of the 20:1 were spun over the flow mold at 1500 rpm for 60 s using a P6700 spincoater from Specialty Coating Systems (Indianapolis, IN, USA). Both were baked in an 80°C oven for 30 min. The control layer was taken off its mold and cut into respective chip pieces. Control line ports were punched using a 20-gauge Intramedic<sup>TM</sup> Luer-Stub adapter (BD Biosciences, Franklin Lakes, NJ, USA). Control layer pieces were washed with ethanol, blown

dry with filtered

air or nitrogen,

and aligned on top

of the flow layer

under a stereo-

scope. The result

was baked in an

80°C oven for 1 h.

Chip pieces were

then cut out and

peeled off the flow

layer mold. Flow

line ports were

punched with the

20-gauge luer-

stub adapter. Chip

pieces were then

washed in ethanol

and blown dry

before binding to

the epoxide glass

slides. The now-

assembled chips



Figure 2. Specificity. Samples each containing 20 nM of a single antigen— CRP (light gray), PSA (black), ferritin (white), and VEGF (dark gray)—were fed in parallel into the test matrix. Each sample produced significant signal above background only in the test corresponding to the antigen contained in the sample. The BSA control produced signal at the background level. The results showed the specificity of measurement and the lack of crosstalk between tests. CRP, C-reactive protein; PSA, prostate-specific antigen; VEGF, vascular endothelial growth factor; BSA, bovine serum albumin.

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underwent a final bake overnight in an 80°C oven (Figure 1A).

#### **Experimental Setup**

An inverted Olympus IX50 microscope (Olympus America, Melville, NY, USA) was equipped with a mercury lamp (HBO<sup>®</sup> 103 W/2; Osram, Munich, Germany), an Olympus Plan 10× objective [numerical aperture (NA) 0.25], a long-distance Olympus SLCPlanFl 40× objective (NA 0.55), a cooled charge-coupled device (CCD) camera (Model SBIG ST-7I; Santa Barbara Instrument Group, Santa Barbara, CA, USA), and a fluorescence filter set (excitation: D540/25, dichroic 565 DCLP; emission: D605/55) from Chroma Technology (Brattleboro, VT, USA). We then plugged 23-gauge steel tubes from New England Small Tube (Litchfield, NH, USA) into the chip's control channel ports. Their other ends were connected through Tygon<sup>®</sup> tubing (Cole-Parmer, Vernon Hills, IL, USA) to Lee-valve arrays (Fluidigm, San Francisco, CA, USA) operated by LabView software on a personal computer. The same types of steel tubes and Tygon plumbing were used to supply reagents to the chip's flow channel ports.

#### **Basic Scheme**

In sandwich immunoassays, a monoclonal antibody, specific to the target analyte (antigen), is bound to a surface. Next, the sample is put in contact with that surface, whereby the antibody captures the contained antigen. Then, a labeled polyclonal antibody attaches to the antigen to complete the "immunostack." The label (e.g., a linked enzyme creating fluorescent product or a fluorophore bound to the polyclonal antibody) generates a signal that is compared with a standard to quantify the captured antigen.

Our chips (Figure 1A) multiplex this scheme to allow 5 simultaneous tests for each of 10 samples. Micromechanical valves (19) direct the pressure-driven reagent flow as desired along a network of 10  $\mu$ m-tall channels (Figure 1B). The "four-way" valving at each intersection in the test

### **Research Report**

matrix forms a capture microchamber, within which the immunoassay stack is built for a particular sample-test combination (Figure 1C). Figure 1D shows a typical fluorescence image from such a microchamber formed by 20  $\mu$ m-wide channels (here, a VEGF test of 0.3 nM sample).

In a typical experiment, monoclonal antibodies flow from D1-5 (derivatization inputs) to DE1-5(derivatization exhausts) in Figure 1B. The antibodies covalently bond to the epoxide floor of the microchannels, producing the first layer of the immunostack. Tris buffer from DB (derivatization buffer input) to DE1–5 removes unbound excess protein and passivates any unreacted epoxide moieties that would otherwise produce background by binding protein in later feeds. Next, Tris buffer flows from SB (samples buffer input) to SE1–10 (samples exhausts) to passivate the rest of the microchannels.

As samples flow in parallel from S1–10 (samples inputs) to SE1–10, each sample fills a corresponding pair of microchannels. When the appropriate valves are closed, each such pair forms a circular path (a coliseum) that traps 10 nL of the respective

sample. Then, an array of peristaltic micropumps (19) drives each trapped volume around its coliseum, with a lap time of 20 s (Figure 1C). Within each coliseum, each antigen is captured in its respective microchamber, as determined by the first layer of the immunostack. The same sample is allowed to run multiple laps (typically 10) to maximize extraction of the antigen from the sample.

After harvesting, buffer from SB to SE1–10 flushes out the sample volume. Parallel feeds of biotinylated antibodies from A1–5 (antibody inputs) to DE1–5 build up the third layers of the



**Figure 3. Sensitivity.** Twenty samples of known concentrations were tested in two experiments to produce calibration curves for four antigens: (A) PSA, (B) VEGF, (C) CRP, and (D) ferritin. These blood analytes are related to inflammation, prostate cancer, long-term iron buildup, and cancer, respectively. The system demonstrated sensitivity at the clinically relevant abundances (with a signal-to-noise ratio >8 at as low as 10 pM) while using only 100 nL per sample for all tests and only 300 nL of antibody per test for all samples. PSA, prostate-specific antigen; VEGF, vascular endothelial growth factor; CRP, C-reactive protein.

immunostacks in each microchamber. Buffer from DB to DE1–5 removes unattached antibody. Fluorescently labeled streptavidin in PBS buffer flows from SA (streptavidin input) to DE1-5. Buffer from DB to DE1-5 removes unattached excess. All valves are then closed, and fluorescence detection is conducted at each microchamber using an inverted optical microscope and an inexpensive, cooled CCD camera.

#### RESULTS

Blood proteins were chosen to validate the system because blood tests are one of the most common and clinically important applications of immunoassays. In particular, CRP, PSA, ferritin, and VEGF were selected due to their significance in medical diagnostics, the wide concentration range spanned by their clinically normal levels, and the commercial availability of well-validated antigens and antibodies.

To test the specificity of the system, we processed one load of 10 nL for each of four samples, each containing 20 nM of one of the antigens in PBS 0.1% BSA, in a chip with 100 µm-wide channels (approximately 50,000 µm<sup>2</sup> per microchamber). Because every test lane intersects every coliseum in a pair of microchambers, the fluorescence signals of each such pair were added to produce the signal for the respective sample-test combination. After normalizing for area, we divided each signal by the fluorescent background of the particular test as measured in regions unexposed to antigen. Results (Figure 2) show that every sample produced significant signal above background only in the test chambers corresponding to the antigen it contained. In addition, the presence of the antigens does not increase the background in the control case, where PBS 0.1% BSA replaced the antibody feeds. These results demonstrate the specificity of the system.

To test the sensitivity of the system, we ran 10 samples against the same four tests but in devices with 20  $\mu$ m-wide channels at the intersections (approximately 2000  $\mu$ m<sup>2</sup> per micro-chamber). One sample was a control

containing no antigen. Each of the other nine samples contained all antigens at the same concentration, which was varied between 30 pM and 10 nM from sample to sample, all in PBS 0.1% BSA. We processed 100 nL per sample (10 loads of 10 nL). The signal for each sample-test was extracted from fluorescence images of the chambers by subtracting the local background for each image and adding the two such results per sample-test combination. Then, for each test, the signal of the control sample was subtracted from the signals of the other nine samples to produce the final results for each test. To establish reproducibility, the same experiment was repeated in another chip with a new dilution of reagents. Also, the concentration range was expanded (10 pM to 100 nM). Data analysis was conducted as described above. The results were combined in a single plot per test (Figure 3), including the clinically relevant levels (www. labtestsonline.org). The data demonstrate the reproducibility of results in the system.

The net signal for the lowest concentration (10 pM) for each test was divided by the uncertainty of the respective control signal to produce a measure of the observed signal to noise. The results were 164 (CRP), 38 (PSA), 11 (ferritin), and 8 (VEGF).

The PSA test shows a linear calibration between 100 pM and 30 nM (Figure 3A). This dynamic range includes the "gray zone" at 4.0–10.0 ng/mL (133–332 pM). The higher the concentration above the "gray zone," the stronger the indication for prostate cancer. Similarly, VEGF has a linear calibration between 10 pM and 10 nM (Figure 3B). This range includes the important cutoff at 25 ng/mL (0.1 nM), exceeding which is an indication for cancer (21).

The CRP test shows that the system is linear between 10 and 300 pM, after which the signal saturates (Figure 3C). In this case, the sensitivity is excessive since the clinically abnormal levels are above 1.2 mg/dL (110 nM), indicating acute infection. Similarly, the ferritin detection (Figure 3D) is sensitive within and below the normal range of 30–300 ng/mL (60–630 pM) but saturates above it, where long-term iron buildup is indicated.

#### DISCUSSION

The observed saturation for CRP and ferritin can be avoided in a number of straightforward ways, producing devices customized to a particular set of tests. In such chips, the scarce-agent tests would retain the smallest channels for maximal sensitivity, while the abundant-agent tests would have wider channels to increase capture area and thus raise the saturation point. Tests for ultra-abundant agents (e.g., ceruloplasmin, normally at 21-50 mg/dL) would be organized in another section of the chip and would be preceded by a dilution stage to reduce the concentration into the measurable range. Because the dilution factor would be predetermined by the device geometry. straightforward multiplication would yield the correct final result.

The parsimony of the system is important in any immunoassay application where sample is costly or scarce. In blood tests, the current practical requirement is 0.5–2 mL per sample per test, necessitating drawing blood from the vein and making common blood tests difficult for pediatric patients. In contrast, the system presented here uses 100 nL of each sample for all tests, thus enabling the development of portable apparatuses conducting common blood tests by a finger prick.

Simultaneously, the system uses 300 nL (as low as 0.8 ng) of antibody per sample-test combination. In contrast, the state-of-the-art Elecsys<sup>®</sup> PSA kit from Roche Applied Science (Indianapolis, IN, USA) uses 200 ng per sample-test, or 250 times more. The savings have direct consequences in modern healthcare and biomedical research.

In further developments, the produced calibration curves would be used as the established dependences, which allow internal recalibrations to be constructed within each measurement by running just a few reference samples per device. This technique would eliminate systematic sources of variation, such as quality and condition of reagents, intensity of the illumination source, and differences in storage and handling. Simultaneously, the results would be extended to more complex media, such as human serum, plasma, spinal fluid, and biopsy samples. Finally, the test matrix could be expanded to  $50 \times 50$  in commercial products (20).

#### Conclusions

The presented work demonstrates the reduction of immunoassays to a microfluidic high-throughput multiantigen format. The developed system is an important step toward derivative immunoassay applications in scientific research and point-of-care testing in medicine.

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#### COMPETING INTERESTS STATEMENT

S.R.Q. holds equity in a company that has a potential interest in licensing the described technology. The other authors declare no competing interests.

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