# A resistive-pulse sensor chip for multianalyte immunoassays

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MultiAnalyte immunoassays are often required to diagnose a pathologic condition. Here, we show how resistive-pulse sensing and multiple artificial pores can be integrated together on a single chip to detect different antigens rapidly and simultaneously. We use multiple pores on a single chip to detect the size change of latex colloids upon specific antigen–antibody binding on the colloid surface. As a proof-of-principle, we demonstrate our ability to detect simultaneously human G-CSF and GM-CSF antigens on a single chip. Our novel technique is a scalable technology that can lead to the sensing of at least $N^2$ antigens simultaneously with an $N \times N$ array of pores on a single chip.

## Introduction

Detecting specific antigens in human serum is a necessary step for health-care providers in their quest to diagnose, monitor, and understand a potential illness. In certain cases, only a combination of different immunoassays can reveal the presence of a pathologic condition. Examples of multiple immunoassays for disease determination include the *triple* or *quadruple test* of prenatal maternal serum required for the detection of Down's syndrome[1] and the multianalyte serum analysis approach to detect myocardial infarction.[2]

Current multianalyte techniques are based on the long-established sandwich immunoassay in which a capture antibody is used to immobilize a specific antigen on the assay surface and a chemically- or biologically-labeled detection antibody is used to mark the presence of the antigen (Fig. 1a–1d). Multianalyte techniques, however, differ from traditional sandwich immunoassays in their need for separate test zones—one per each particular antigen—and for the use of two or more labels (one per analyte).[3,4] Just as conventional sandwich immunoassays have a number of drawbacks, including lengthy incubation times for functionalizing the assay surface with the capture antibodies (typically 15 h[5]) and the need to label the detection antibodies (which incurs additional steps and reagent use), current multianalyte detection schemes have drawbacks as well. For example only fluorescent labels having different emission wavelengths or kinetics can be employed; as another example, multiple incubation steps are often required. Clearly, there is a compelling need to develop new methods for sensing multiple antigens simultaneously.

In this paper, we demonstrate, for the first time, the ability to perform multianalyte immunoassays using a combination of multiple artificial pores and resistive-pulse sensing on a single chip. Previously, we have shown that resistive-pulse sensing is highly effective in circumventing the drawbacks of traditional sandwich immunoassays:[6,7] incubation times are significantly reduced and labeling is not necessary because the technique is

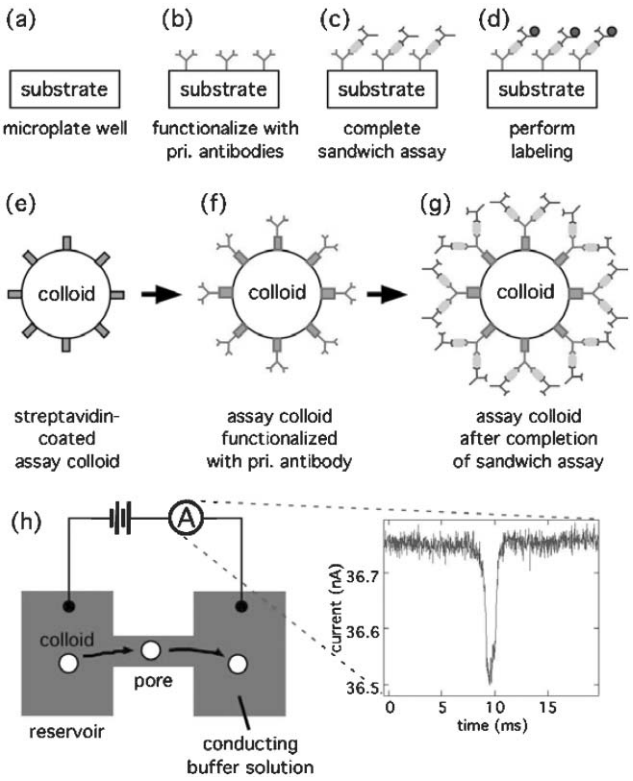


**Fig. 1** Immunoassay techniques. (a)–(d) Conventional ELISA sandwich assay. (e)–(h) Sandwich assay performed on the surface of a colloid. For an ELISA sandwich assay, a microplate well (a) is coated with primary antibodies (b), and then antigens and secondary antibodies are added (c). Labeling the secondary antibodies indirectly reveals the presence of the antigens (d). Streptavidin-coated assay colloids (e) are functionalized with biotinylated primary antibodies which increase the size of the colloids (f). In the presence of specific antigens, secondary antibodies are bound to the colloids, further increasing colloid size (g). Schematic of a resistive-pulse sensor that measures colloid diameter (h). When a colloid passes through a pore with diameter comparable to that of the colloid, a decrease in current is measured. The magnitude of the current pulse is related to the colloid diameter through eqn (1).

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purely electronic and therefore label-free and direct. Here, we show that resistive-pulse sensing can be used to detect simultaneously two different human antigens on a single chip. Specifically, we demonstrate that we can detect concurrently human granulocyte and macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF), both of which are associated with acute inflammation[8] and can be involved with tumor progression.[9–11] While we have chosen GM-CSF and G-CSF detection as our proof-of-principle, we emphasize that resistive-pulse sensing applies to a wide variety of ligands that can be detected simultaneously, thus making this technique a key platform technology for multiple immunoassays.

## Theory

The resistive-pulse technique of particle sizing[12] centers on a particle passing through a pore and displacing conducting fluid in that pore. This leads to a transient increase, or pulse, in the pore's electrical resistance that in turn is measured as a decrease in current (see Fig. 1h). For a particle smaller than that of a pore, the relative current change of the particle flowing through the pore is

$$\left|\frac{\delta I}{I}\right| = \frac{D}{L}\left[\frac{\arcsin\left(d/D\right)}{\sqrt{1-\left(d/D\right)^2}} - \frac{d}{D}\right] \quad (1)$$

as predicted by Deblois and Bean.[13] Here, $d$ is the particle diameter, $D$ is the pore diameter, and $L$ is the pore length. For simplicity, eqn (1) can be approximated as the volume ratio of particle to pore: $|\delta I/I| \sim V_{\text{particle}}/V_{\text{pore}}$.

As we have demonstrated previously,[6] the resistive-pulse sensing technique can be employed as an electronic immunoassay that can detect in a label-free and direct manner the presence of antigen in solution. The basis of this immunoassay is that of detecting the increase in diameter of a sub-micron latex colloid upon binding to an unlabeled specific antibody (see Fig. 1e–1g). Using micron-sized pores embedded in polydimethylsiloxane (PDMS), we have successfully employed this novel technique to perform two important types of immunoassays: an inhibition assay, in which we detect the presence of an antigen by its ability to disrupt the binding of antibody to the colloid; and a sandwich assay, in which we successively detect the binding of each antibody in a two-site configuration.[7]

## Method and procedures

In this section we describe the design and fabrication of the parallel artificial pores we employ for multianalyte sensing. Furthermore, we present the colloid preparation and the electronic instrumentation necessary for performing the simultaneous detection of two antigens.

### Device design

Each device, which we refer to as dual-pore sensor, consists of a set of two pores (10 μm-long × 1 μm-wide × 1 μm-high) that are connected on either end to two reservoirs (600 μm-long × 200-μm wide × 7 μm-high). 1 μm-wide filters are included at the entrance of each reservoir to prevent dust or other unwanted material from clogging the pores. In addition, two pairs of electrodes are positioned on either side of the pores for measurement. Fig. 2 shows the layout of the device used for this study.

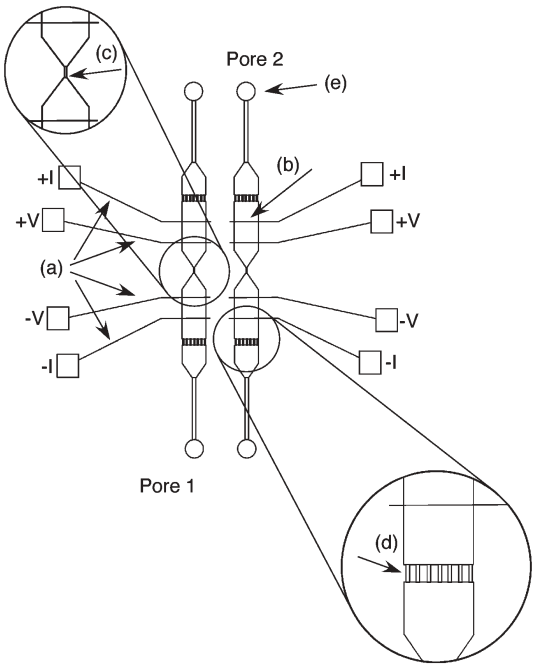



**Fig. 2** Dual-pore device design. (a) Four-terminal electrode layout. (b) 600 μm-long × 200 μm wide × 7 μm-high reservoir. (c) 10 μm-long × 1-μm-wide × 1 μm-high pore. (d) 20 μm-long × 1-μm-wide × 1 μm-high pore filters. (e) Inlet and outlet ports.

### Fabrication of electrodes

Pairs of Pt/Ti electrodes (50 Å/250 Å) are fabricated on a glass wafer using traditional photolithography and thin-film deposition techniques. The glass wafer is then diced into individual chips, which are subsequently ready for PDMS sealing.

### PDMS molding

Soft lithography is used to cast the pores and the reservoirs into a polydimethylsiloxane (PDMS) slab (Fig. 3a–3b).[14] Specifically, a negative relief master of an array of pores (10 μm-long × 1 μm-wide × 1 μm-high) is created on a Si wafer using electron-beam lithography and $SF_6$ reactive-ion etching. The reservoirs are subsequently optically patterned onto this master. PDMS (10 : 1 prepolymer : curing agent) is then dispensed onto the completed master and cured overnight at 80 °C. Individual sets of pores are cut and removed for sealing onto the previously-patterned glass substrates. Inlet and outlet holes are punched through the reservoirs using a 16 G syringe needle.

### Pore sealing

The glass substrate and the PDMS slab embedded with pores and reservoirs are both treated with an oxygen plasma

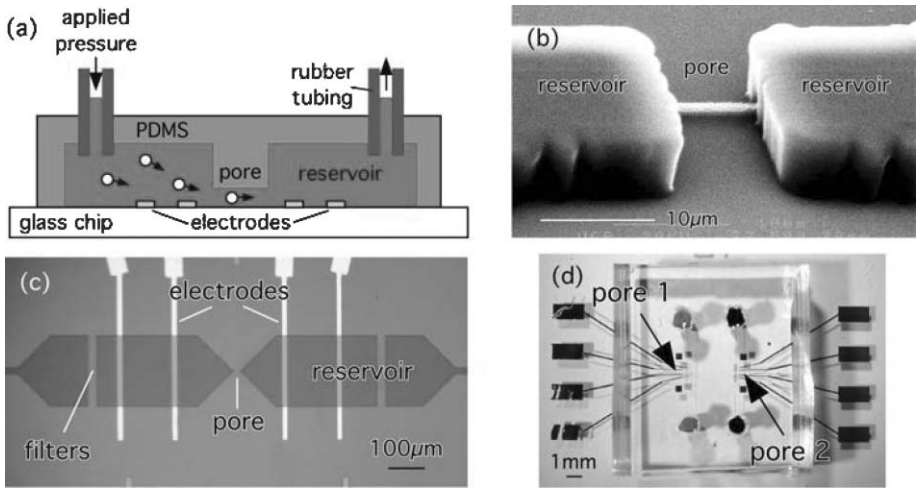



**Fig. 3** Design of resistive-pulse sensor. (a) Side-view schematic of a single pore. A molded PDMS slab containing the reservoirs and pore is sealed to a glass chip with prefabricated Ti/Pt electrodes. (b) SEM picture of master mold highlighting details of the pore and reservoirs. (c) Optical view of completed sensor as viewed from the top. Microfluidic channels have been filled with dye for enhanced visibility. (d) Photograph of dual pore sensor containing two separate pores on a single chip.

(200 mTorr, 5 mA, 10 s). The substrate and PDMS slab are then aligned under an optical microscope and permanently sealed together on a hotplate (10 min at 65 °C followed by 15 min at 150 °C). Fig. 3c is an optical image of a completed pore sensor showing the arrangement of the electrodes, reservoirs, and pores. Shown in Fig. 3d is the dual-pore device that enables two separate immunoassays to be performed simultaneously on a single chip.

### Electronic apparatus

A four-terminal electronic measurement of each pore is conducted using the pairs of Pt electrodes previously fabricated on the glass substrate. A four-terminal measurement is necessary in order to remove the resistance of the electrodes and the interfacial resistance between the electrodes themselves and the buffer solution.[6] Thus, only the resistance of the pore is measured. A fixed voltage of 0.70 V is maintained across both pores using two Stanford Research Systems DS345 function generators. Current passing through the pores is amplified with two DL Instruments 1211 current preamplifiers and sampled with a National Instruments PCI-6035E data acquisition card (Fig. 4) connected to both preamplifiers. Data are recorded and analyzed using custom-written software.

### Colloid preparation and experimental procedure

The assay colloids used in these experiments are 490 nm streptavidin-coated colloids from Bangs Labs (Fishers, IN), and the reference colloids used to calibrate the pores are 470 nm sulfate-coated polystyrene colloids from Interfacial Dynamics (Portland, OR). While the assay colloids are diluted to a working concentration of $1.4 \times 10^9$ colloids $mL^{-1}$ (1 : 100 dilution), the reference colloids are diluted to $1.2 \times 10^9$ colloids $mL^{-1}$ (1 : 1000 dilution). The two different colloids are subsequently mixed in a buffer solution consisting of 0.5× phosphate buffered saline, 1 mg $mL^{-1}$ bovine serum albumin (as a blocking agent), and 0.1% (v/v) Tween 20 (as a surfactant). Prior to use, both colloids are washed in buffer by centrifugation (8700 rpm for 14 min, repeated a total of three times).

Multinalyte assays are performed simultaneously for human GM-CSF and human G-CSF antigens. The capture and unlabeled detection antibodies, as well as the reference antigens for both assays, are obtained from ELISA DuoSet kits (R&D Systems, Minneapolis, MN). To detect the antigens, the assay colloids are incubated with 10 μg $mL^{-1}$ capture antibodies for 15 min to thus functionalize them. The colloids are then thoroughly washed to remove the excess antibodies from solution. The assay colloids conjugated to primary antibodies are injected into the dual pore device connected to the electronic apparatus described above and an external pressure (1–3 psi) is applied to drive the colloids through each pore. The change in resistance due to a colloid passing through the pore is measured and eqn (1) is used to determine the mean colloid diameter.[6,7,15] The same functionalized assay colloids are then incubated with both the antigens and detection antibodies for 20 min to complete the sandwich

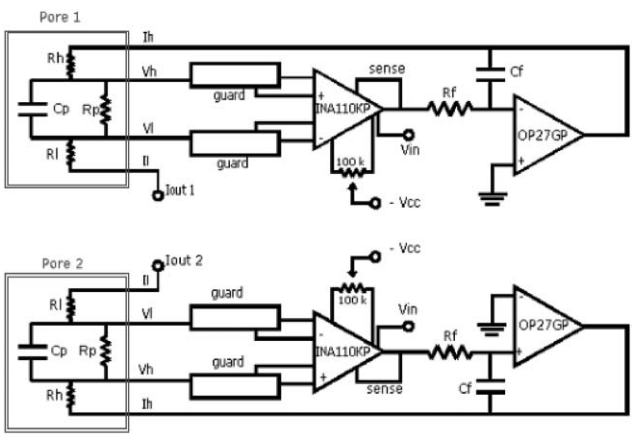



**Fig. 4** Circuit used to perform a fixed voltage four-terminal electronic measurement of each pore.

assay and the same measurements are repeated. Size changes corresponding to bound GM-CSF and human G-CSF antigens are thus detected.

To improve the resolution of all measurements, the reference colloids of fixed size are measured together with the assay colloids to thus calibrate the diameter of the pore and remove variations in pore diameter arising from device fabrication. Furthermore, colloids that travel off the central axis of the pore are taken into account. As we have described previously,[15] relative to particles that travel on-axis, off-axis particles take longer to transit the pore (causing wider pulses) and produce greater current changes. The former effect, which we refer to as hydrodynamic off-axis effect, is due to the parabolic distribution of fluid velocity within the pore. The latter effect, which we refer to as electrical off-axis effect, occurs because off-axis particles enhance the non-uniformity in the distribution of the electrical current density, leading to a further increase in the electrical resistance.[16] We have recently developed an algorithm for removing these off-axis effects:

$$\left|\frac{\delta I}{I}\right|_{\mathrm{adj}} = \left|\frac{\delta I}{I}\right| - (f(\tau) - f(\tau_{\min})) \qquad (2)$$

where $\tau_{\min}$ is the minimum transition time of the on-axis particles and $f(\tau)$ is a fitted line to a plot of $|\delta I/I|$ *versus* the transition time $\tau$. In short, eqn (2) is an algorithm to calculate the pulse height each colloid would have caused had it traveled on the central axis of the pore.

## Results and discussion

In this section, we report on the simultaneous assay of different human antigens on a single chip to demonstrate, for the first time, the scalability of resistive-pulse sensing. Fig. 5 shows representative data measured simultaneously in Pores 1 and 2 of a dual-pore device. Each pulse in the current trace corresponds to a single functionalized colloid passing through a pore. We used Pore 1 to perform an immunoassay of human GM-CSF antigens, and Pore 2 for human G-CSF. Fig. 6a is a typical histogram of the normalized pulse height ($|\delta I/I|$) measured for a solution containing both the assay and reference colloids. As shown, there are two distinct clusters of colloids. Fig. 6b shows the normalized pulse height data before and after applying the correction (eqn (2)) for off-axis effects. As shown, the dotted line represents the raw data while the solid line shows the same data after correction. The change is minimal because the diameter of the colloids used is comparable to the diameter of the pore. Fig. 7 shows the results of several simultaneous immunoassays. The data in columns B–D corresponds to the result of two measurements

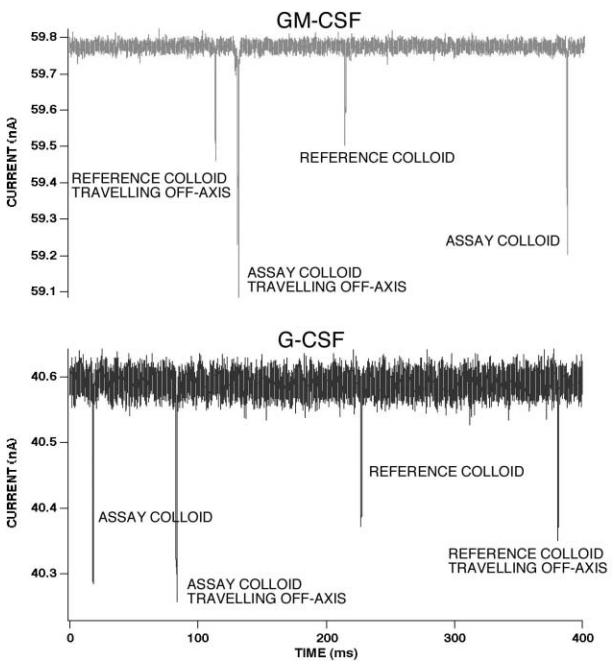



**Fig. 5** Representative data from Pore 1 and Pore 2 measured simultaneously in the dual pore sensor. Each pulse in the data corresponds to a single colloid. Pore 1 was used to assay GM-CSF antigens while Pore 2 was used to assay G-CSF antigens.

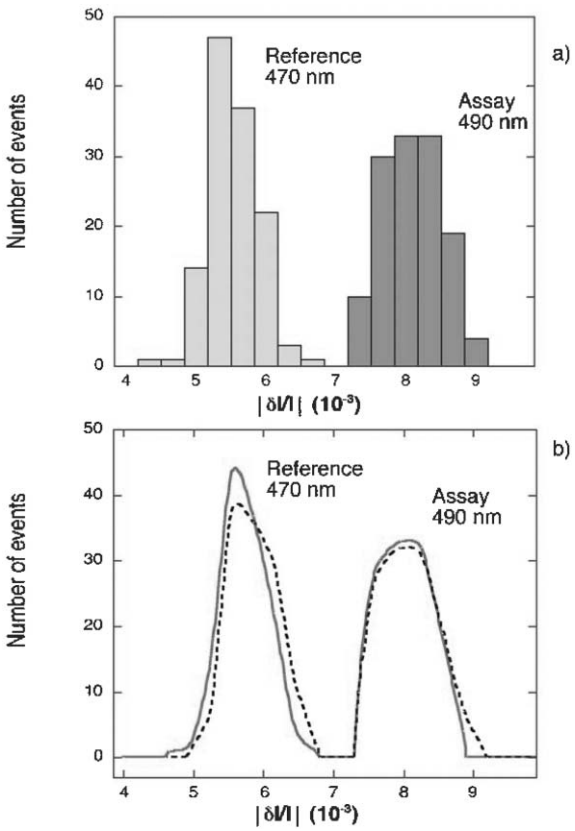



**Fig. 6** Data analysis and correction of off-axis effect. (a) Histogram of the normalized pulse heights $|\delta I/I|$ showing two distinct populations of colloids flowing through the same pore: reference colloids (470 nm) and assay colloids (490 nm) conjugated with primary antibodies G-CSF. A pressure of 2.25 psi was used in this experiment and the measured values of $D$, the pore diameter, and $d$, the assay colloid diameter, as determined by the data and eqn (1) were 1.18 μm and 518.45 ± 0.63 nm respectively. (b) Histogram of the normalize pulse heights $|\delta I/I|$ before and after applying the correction for off-axis effects (eqn (2)). The dotted line represents the raw data, while the solid line shows $|\delta I/I|$ after correcting for the effects of off-axis colloids. As shown, the application of this correction leads to a decrease in the coefficient of variation of the pulses measured from 6% to 5.8% for the reference colloids and from 5% to 4.9% for the assay colloids.

performed in parallel. The data in column A corresponds to the diameter of the unfunctionalized assay colloid (512.2 nm). The presence of different components in each measurement is indicated by a checkmark under each bar.

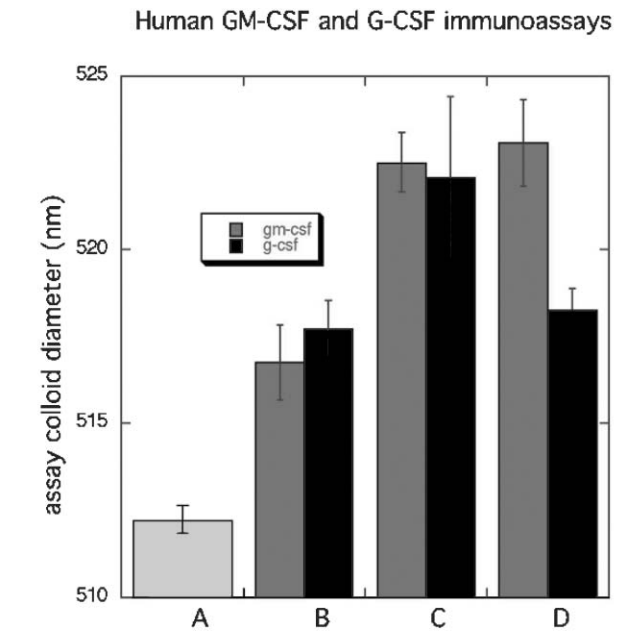
Column B shows the diameters of assay colloids after functionalizing them with human GM-CSF (left) and G-CSF (right) primary antibodies. The colloids were incubated with a buffer solution containing 10 μg $mL^{-1}$ of biotinylated primary antibodies. With this working concentration, assay colloids in both cases showed a size increase of 5 nm over the plain assay colloids (column A).

In column C, sandwich assays for GM-CSF (left) and G-CSF (right) were completed.[17] As indicated, the diameter of both sets of assay colloids increased by 5 nm over the functionalized colloids (column B) to 522 nm. This demonstrates that both assays positively detected the presence of their respective antigens (GM-CSF at 84 ng $mL^{-1}$ and G-CSF at 90 ng $mL^{-1}$). The 5 nm diameter increase here suggests that as many secondary antibodies as primary antibodies became attached to the colloid. The volume added by the antigens is not expected to contribute significantly to the diameter increase as the antigen is about 10× less massive than the antibody.[18]

In the final column, column D, a control experiment was performed in which GM-CSF antigen (84 ng $mL^{-1}$) was added to the GM-CSF sandwich assay (left), but non-specific GM-CSF antigen (84 ng $mL^{-1}$) was added to the G-CSF sandwich assay (right). The assay for GM-CSF showed a clear size increase of 5 nm over the functionalized assay colloids (column B), but the assay for G-CSF did not. This shows that size increase of colloids is measured only if specific antigen–antibody binding occurs. If this condition is not satisfied, antigens are not sensed.

The antigen concentrations detected are 84 ng $mL^{-1}$ and 90 ng $mL^{-1}$ for GM-CSF and G-CSF, respectively. Since the standard deviation of the colloid diameter is 13 nm, we can achieve a resolution of 0.2% if 200 or more colloids are measured during each experiment. This corresponds to our measuring a change in diameter as small as 1 nm in a 512 nm colloid and to our detecting a concentration as little as 15 ng $mL^{-1}$. To further increase the sensitivity of our device, we suggest decreasing the colloid concentration or using smaller or monodisperse colloids.

Although we have performed the necessary chemistry and incubation steps outside the chip, the microfluidic platform we have used for our dual pore sensor can readily include reservoirs and mixers for a Micro Total Analysis System (μTAS). In addition, although we demonstrated the simultaneous detection of two antigens, ours is a scalable technology that can lead to the sensing of a greater number of antigens simultaneously. Since colloids with different diameters generate pulses with different magnitudes (see Fig. 5), several antigens can be detected in the same pore by assigning them to colloids of different diameters. Currently we are capable of sensing up to four differently-sized colloids (370, 460, 560, 640 nm) in a single pore.[6] Since the change in diameter due to the binding of antibodies is on the order of 5–10 nm, we should be able to discern readily the pulses generated by the respective colloids without being concerned that the pulses from one colloid size could be attributed to those of another colloid size. Given this and what we have demonstrated in this paper, it is thus possible to perform at least $4N^2$ simultaneous assays on a single chip of $N \times N$ arrays of pores. This level of multiple detection is truly unprecedented.




| | A | B | | C | | D | |
|---|---|---|---|---|---|---|---|
| assay colloid | √ | √ | √ | √ | √ | √ | √ |
| GM-CSF pri ab | | √ | | √ | | √ | |
| G-CSF pri ab | | | √ | | √ | | √ |
| GM-CSF antigen | | | | √ | | √ | √ |
| G-CSF antigen | | | | | √ | | |
| GM-CSF sec ab | | | | √ | | √ | |
| G-CSF sec ab | | | | | √ | | √ |

**Fig. 7** Assay colloid diameter measured under a variety of conditions. In column A, the diameter of the unfunctionalized assay colloid is indicated. In each of columns B–D, two separate but simultaneous immunoassays were conducted, one for GM-CSF antigen and the other for G-CSF antigen. In column B, the diameters of assay colloids functionalized with GM-CSF and G-CSF primary antibodies are shown. Both measurements showed clear increases in diameter of 5 nm as compared to the unfunctionalized assay colloid. In column C, immunoassays of GM-CSF and G-CSF antigens were completed, resulting in a further 5 nm increase in the diameter. This indicates that both assays detected the presence of their respective antigen. In column D, GM-CSF antigens were used in both assays, resulting in a diameter increase (compared to column B) in the GM-CSF assay, but not in the G-CSF assay. This shows that specific binding between antigen and antibodies is required for the size increase and antigen detection.

## Conclusions

With this work, we address the compelling need to detect simultaneously different antigens that play a concurrent role in many pathologic situations. In this paper, we present a device designed to perform multianalyte detection using multiple pores and the resistive-pulse sensing technique. Our device, consisting of two artificial pores, permits one to overcome the drawbacks that are encountered when performing

immunoassays using more traditional methods. The design and the fabrication process of the chip as well as the electronic apparatus employed are described in depth. Using a dual-pore device, we have demonstrated, for the first time, the simultaneous immunoassays of two different human antigens on a single chip. Because of the scalability of our technology and its ability to perform rapidly and label-free, we believe that our device represents a new direction in the development of high-throughput devices for immunology.

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17 This concentration corresponds to $4 \times 10^3$ IgG antibodies $mL^{-1}$. Assuming that the surface area occupied by each antibody is $\sim 65$ $nm^2$,[19–21] $\sim 12\,000$ biotinylated antibodies can be bound to each assay colloid. This number corresponds to a theoretical saturation concentration of antibodies of 3.0 μg $mL^{-1}$. An excess of antibodies incubated (10 μg $mL^{-1}$) was used to ensure the maximum coverage of the colloid surface. Although the 5 nm diameter increase of the assay colloids when measured with primary antibodies is less than the actual molecular size of IgG (most likely due to the antibody conformation on the colloid surface), the validity of our immunoassay is still inarguable.
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