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Microfluidic Communicating Vessel Chip for Expedited and Automated Immunomagnetic Assays

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Abstract

Rapid, sensitive analysis of protein biomarkers is of tremendous biological and clinical significance. Immunoassay is a workhorse tool for protein analysis and has been under continuous investigations to develop new methods and to improve the analytical performance. Herein we report a pneumatically gated microfluidic communicating vessel (μ COVE) chip for rapid and sensitive immunomagnetic ELISA. A distinct feature of our device is that it employs the communicating vessel principle as a simple means to generate fast transient hydrodynamic flow to enable effective flow washing without the need of excessive incubation, which greatly simplifies and expedites the assay workflow, compared to conventional microfluidic flow-based immunoassays. The stationary multi-phase microfluidic techniques have been developed for fast bead washing. However, they have some limitations, such as the need of careful control of interfacial properties, large bead quantity required for reliable interphase bead transport, and relatively high bead loss during surface tension-gated traverse. Our signal-phase μ COVE chip can overcome such limitations and facilitate the manipulation of magnetic beads to streamline the assay workflow. We showed that the μ COVE device affords highly sensitive quantification of CEA and EGFR proteins with the LOD down to the sub-picogram per mL level. Direct detection of EGFR in the crude A431 cell lysate was also demonstrated to further validate the ability of our device for rapid and quantitative analysis of complex biological samples. Overall, our work presents a unique platform that combines the merits of the stationary multi-phase systems and the flow-based microfluidics. This novel immunoassay microsystem holds promising potential for a broad range of biological and clinical applications, owing to its simplicity and high performance.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Introduction

Immunoassay based on specific antibody-antigen interactions has been one of the most versatile and widely used bioassays in chemistry, life sciences, and medicine¹. Immunoassay enables sensitive and specific measurements of a broad spectrum of targets, ranging from small molecule compounds (e.g., drugs and toxins)² to macromolecules like peptides and proteins, to bioparticles such as extracellular vesicles and virus^{3–5}, and to bacterial and mammalian cells^{6, 7}. Microfluidics is well poised to develop the new-generation immunological technologies because of its unique advantages in combining precise flow control, promoted biochemical reactions in small volumes, circuit-level integration, and system automation^{8, 9}. Numerous microfluidics-based immunoassays have been developed to improve analysis sensitivity, speed, and multiplicity^{9, 10}; and the technologies are continuously evolving, driven by the pressing needs of better analytical tools for emerging applications ranging from single-cell analysis^{11–14} to point-of-care diagnosis^{15, 16} and to precision medicine^{17, 18}.

A typical protocol of conventional immunoassay, such as enzyme-linked immunosorbent assay (ELISA), encompasses a series of washing, mixing, and incubation steps, which consume large volumes of reagents and are labor intensive and time consuming and often takes several hours or even up to 2 days. Standard immunoassay protocols can be substantially simplified and expedited by leveraging microfluidic technology to accelerate and minimize processing steps. For instance, hydrodynamic flow washing in microchannels obviates the need for repetitive washing steps with large volumes of buffers required by microplate ELISA to effectively reduce non-specific background¹⁹. Moreover, several wash-free immunoassays have been developed with different microfluidic techniques, including a microfluidic droplet splitting system coupled with surface-enhanced Raman scattering (SERS) detection²⁰, an integrated microfluidic homogeneous AlphaLISA chip²¹, and a microfluidic giant magnetoresistive (GMR) biosensor platform²². Eliminating excessive washing in the immunoassay protocol affords significant advantages in improving analytical performance as it 1) simplifies and expedites the assay by removing multiple time-consuming steps; 2) avoids potential variation in analysis, and 3) enhances detection sensitivity by minimizing dissociation of analyte, especially weakly bound protein complexes. Despite these benefits, the aforementioned wash-free microfluidic methods require sophisticated chip design, fabrication, and operation expertise, which limit their broad applications.

Magnetic separation of analyte binding beads is a versatile method for sample preparation and bioassays because of its simplicity and ease of automation²³. Combining magnetic bead manipulation and unique microscale surface effects, a multi-phase microfluidic strategy has been recently developed to largely simplify and expedite the workflow of bioassays. In this strategy, the aqueous assay reagents are dispensed into an array of stationary droplets^{24–28}, open reservoirs^{29–32}, or microchambers³³ isolated by an immiscible phase, e.g., oil or air, and external magnetic field is used to pull the affinity magnetic beads across these compartments sequentially to complete the sample preparation and/or detection workflow. This stationary multi-phase strategy exploits the surface tension-caused volume pinning effect to separate analyte-bound beads from the bulk media with small-volume of carryover, which enables rapid and automated bioassays without tedious washing. Despite their advantages, the stationary multi-phase microfluidics requires tight control of the properties of interfaces between the phases and demands a large amount of beads to generate sufficient magnetic forces to overcome surface tension. Bead loss may occur during such surface tension-gated traverse. Moreover, small carryover of unbound materials by unwashed bead clusters can result in non-specific signals that can limit the analytical sensitivity or cause false positives in clinical applications, especially for extremely sensitive amplification-based assays.

Distinct from the previous microfluidic immunoassays implemented with either flow-based or stationary multi-phase microsystems, herein we report a single-phase, pneumatically gated microfluidic communicating vessel chip (μ COVE) for simplified and expedited immunoassays (Fig. 1). First, while our device adapts the similar format of reagent loading and immunomagnetic assay workflow for the stationary multi-phase microsystems, an array of pneumatically actuated gates instead of an immiscible phase is used for the compartmentalization of aqueous reagents. Thus, our single-phase platform takes advantage of stationary microfluidics to simplify and streamline the assay workflow, while avoiding the aforementioned limitations associated with the multi-phase operation. Furthermore, in contrast to the stationary assay systems, μ COVE employs hydrostatic pressure-driven flow, the simplest approach for fluid control in a microfluidic system, to afford rapid yet effective washing of immunomagnetic beads. Overall, our device based on the communicating vessel principle presents a novel immunoassay platform that combines the advantages of the stationary multi-phase microfluidics in simplicity and streamlined operation and the benefits of effective washing provided by the flow-based microfluidics. As of a proof-of-concept, we validate our platform by detecting two protein biomarkers commonly targeted for cancer diagnosis, carcinoembryonic antigen (CEA) and epidermal growth factor receptor (EGFR). Our results demonstrated highly sensitive detection of these biomarkers down to the level of sub-picogram per mL. Direct measurement of the biomarker expression in crude A431 cell lysate using different washing methods further verifies the ability of the μ COVE device for rapid immunoassay without excessive washing steps. Owing to its simplicity and high performance, our system would provide a useful platform for clinical analysis of protein biomarkers to facilitate disease diagnosis and other applications such as point-of-care (POC) testing.

Materials and Methods

Chip Fabrication.

Two-layer polydimethylsiloxane (PDMS) microchips (Fig. 1A) were fabricated using standard multilayer soft lithography. In brief, silicon wafers were cleaned with piranha solution (75% sulfuric acid and 25% hydrogen peroxide), and coated with SU-8 photoresist (MicroChem) by spin-coating. The SU-8 microstructures were fabricated onto the wafers from the photomasks designed with AutoCAD, following the protocols recommended by the manufacturer for fabricating 50 μm thick bottom flow layer (SU-8 2025) and 100 μm thick top pneumatic layer (SU-8 100), respectively. The SU-8 molds were treated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma-Aldrich) by gas-phase silanization under vacuum for at least 4 hrs. To make the pneumatic layer, PDMS base and curing agent (Dow Corning) were mixed at a 10:1 ratio (35 g in total), poured on the mold and cured in the oven at 70 °C for at least 2 hrs. The PDMS pieces were peeled off from the mold, cut, and punched to make pneumatic connection holes. A thin PDMS flow layer was made by spin coating the mold with 6 g of the prepolymer mixture (15:1) followed by curing at 70 °C for 30 min in an oven. The two layers were treated by UV/ozone briefly, manually aligned under a stereomicroscope, and permanently bonded by baking in the 70 °C oven. The two-layer PDMS slabs were then peeled off the mold and reservoirs were punched. Lastly, an integrated microchip was fabricated by permanent bonding of PDMS slab and glass slide using UV/ozone treatment (Fig. 1B). The assembled chip was blocked by 5% bovine serum albumin (BSA, Fisher Scientific) overnight at 4 °C to reduce non-specific binding.

Magnetic Beads Preparation.

100 μL of approximately 30 mg/mL carboxylic acid coated magnetic beads (Dynabeads M-270 Carboxylic Acid, Invitrogen) were transferred to a 1.5 mL microcentrifuge tube and washed twice with 100 μL of 25 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 5, Sigma-Aldrich) for 10 min. The beads were then activated with 50 μL of freshly prepared 50 mg/mL N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma-Aldrich) and 50 μL of 50 mg/mL N-hydroxysuccinimide (NHS, Sigma-Aldrich) with rotation at room temperature for 30 min. The activated beads were washed twice with 100 μL of 25 mM MES buffer (pH 5) and then mixed with 60 μg of anti-CEA capture antibody (10-C10F, Fitzgerald) or anti-EGFR capture antibody (DYC1854-2, R&D systems), respectively. After overnight incubation with rotation at 4 °C, the beads were magnetically collected to remove supernatant and blocked by 100 μL of 50 mM ethanolamine (Sigma-Aldrich) in phosphate-buffered saline (PBS, Corning Cellgro) for 60 min at room temperature with rotation. Finally the capture antibody labelled magnetic beads were washed four times with 100 μL of washing buffer (0.1% BSA and 0.1% Tween-20 in PBS), resuspended in 200 μL PBS, and stored at 4 °C.

Microchip Immunomagnetic Assay.

Protein standards and detection antibodies were prepared in PBS working solution (PBSW, pH 7.4) containing 1% bovine serum albumin (BSA) (Sigma-Aldrich). A series of protein standards was prepared by 10-fold dilution of the stock protein solution. 0.5 $\mu\text{g/mL}$ streptavidin conjugated β -galactosidase (S β G, Life Technologies) and 0.2–0.5 mM resorufin

β -D-galactopyranoside (RGP, Life Technologies) were prepared in PBSW with 2 mM MgCl_2 (Fluka Analytical) added. For CEA quantification, 3.2 pg/mL to 32 ng/mL CEA protein standards (30–1819, Fitzgerald), 43 $\mu\text{g/mL}$ biotinylated anti-CEA detection antibody (61-C10GB, Fitzgerald) and 0.2 mM RGP were used in the microchip immunomagnetic assays. For EGFR quantification, 0.2 pg/mL to 2 ng/mL EGFR protein standards (DYC1854–2, R&D systems), 3.6 $\mu\text{g/mL}$ biotinylated anti-EGFR detection antibody (DYC1854–2, R&D systems) and 0.5 mM RGP were used. For biological sample analysis, non-denatured A431 whole cell lysate (L015V2, Abnova) was diluted by 1000 and 10000 folds in PBSW and used for the measurement of EGFR expression level.

The on-chip assay was performed following the procedure illustrated in Fig. 1C. The monolithic diaphragm gates were actuated at 90 kPa pressure and -87 kPa vacuum controlled by a homemade solenoid controller via a LabVIEW program. A 5% BSA blocked chip was washed with PBS and loaded with 10 μL assay reagents and 20 μL buffers in the individual vessels as specified as follows. A 20 μL protein sample was mixed with 1 μL of 15 mg/mL capture antibody labelled magnetic beads in the first reaction vessel and incubated for 2 hrs with rotation, during which the vessels were covered by PDMS slabs in order to minimize evaporation. After incubation, the magnetic beads were concentrated into the connecting channel by a magnet and the sample solution was removed by aspiration. With the pneumatic gate opened, the protein/bead complexes were magnetically drawn into the second vessel loaded with 20 μL of washing buffer within ~ 20 seconds. The second gate was then opened and the bead complexes were magnetically pulled into the third vessel containing 10 μL of biotinylated detection antibody and incubated for 1 hr with the gates closed. Following the same operation, the immunocomplex-bound beads were pulled through the next buffer vessel for rapid washing and into the fifth vessel containing 10 μL of 0.5 $\mu\text{g/mL}$ S β G for enzyme tagging. After 30 min incubation, the bead complexes were pulled through two buffer vessels into the detection microchamber and mixed with the RGP substrate loaded from the last vessel by diffusion. Fluorescence detection was performed after 30 min enzymatic reaction. Fluorescence images were acquired using an inverted epifluorescence microscope equipped with a 10 $\times$ objective and a scientific CCD camera (Carl Zeiss) and analyzed with ImageJ (NIH, <http://rsbweb.nih.gov/ij/>) to measure the fluorescence intensity.

Results and Discussion

Chip Design and Assay Principle.

The μCOVE chip is designed to streamline and expedite the multi-step workflow of conventional magnetic bead-based immunoassays. Fig. 1A and B display the prototype μCOVE chip that we have devised. To demonstrate the scalability of our design, the three-layer PDMS/glass chip integrates four parallel μCOVE units, each consisting of seven vessels of ~ 20 μL volume connected by the flow channels (~ 50 μm high) fabricated on the bottom PDMS membrane (~ 200 μm thick). The adjacent vessels are disconnected by the normally-closed gates that can be lifted by the pneumatic channels fabricated on the top PDMS layer. The vessel array is coupled with an enclosed detection microchamber at the end which is connected to a reservoir for loading the enzymatic substrate, RGP (Fig 1B).

The immunoassay workflow based on the μ COVE chip is schematically illustrated in Fig. 1C. Briefly, with all gates closed under pressure, the first vessel is loaded with 20 μ L sample mixed with antibody-coated magnetic beads and others are loaded with either 10 μ L reaction reagents (*i.e.*, detection antibody, S β G, and RGP) or 20 μ L washing buffers. After capturing the target proteins, the beads were pulled down by a magnet and 10 μ L supernatant was removed from the sample vessel. The beads are pulled across a washing vessel with the two controlling gates opened one at a time. Similarly, the beads are then sequentially drawn across the downstream vessels containing biotinylated detection antibody, washing buffer, and S β G to form the sandwiched immunocomplexes. Finally, the beads are pulled across two washing vessels into the detection chamber and mixed with the RGP substrate loaded from the last vessel by diffusion. The tagged enzyme converts RGP to fluorescent resorufin molecules for quantitative measurement of the bead-captured proteins. Thus, the μ COVE chip combines magnetic bead manipulation and pneumatic microvalve actuation, both of which are amenable to microfluidic integration and automation to effectively streamline the workflows for sample preparation and bioassays.

A standard immunoassay protocol requires multiple washing steps that can be tedious. In addition to streamlining the workflow, a distinct feature of our design is that it exploits hydrodynamic flow generated by the communicating vessels to expedite the washing steps and thus the entire assay. Rapid bead washing by hydrodynamic flow in a μ COVE chip, herein referred as to hydrodynamic washing, is conceptually depicted in Fig. 1D. It is well known that in communication vessels, the fluid flows to reach the same level in all of the vessels driven by the hydrostatic pressure $P = \rho g h$, where ρ is the density of the fluid, g is the gravitational acceleration, and h is the difference in elevation. We load the washing buffer vessels to a higher level than the reagent vessels, which creates hydrostatic pressure between two adjacent vessels. Magnetic beads in a reagent vessel can be concentrated at the closed gate using a magnet. When the gate is lifted up, the beads will be pulled through the connecting channel and washed by a counter flow of washing buffer generated by the hydrostatic pressure between the connected vessels. As discussed in details in the following sections, our results showed that the hydrodynamic washing in our μ COVE chip is able to effectively suppress non-specific background without the need to re-disperse and wash the beads in the buffer vessels for an extended period, which largely reduces the overall analysis time (Table 1).

μ COVE Operation.

We first characterized the hydrodynamic flow induced by hydrostatic pressure for bead washing and pneumatically gated magnetic manipulation of beads as they are the key processes of the μ COVE operation. In our device, the vessels of ~ 20 μ L volume were punched through ~ 5 mm thick PDMS chips with a 2.5-mm biopsy punch. A pair of gated communicating vessels were filled with 10 μ L resorufin solution and 20 μ L water, respectively, which are the typical liquid volumes used for the actual immunoassay. The normally closed pneumatic gates were found to completely block the flow between two adjacent vessels, indicated by no observed transport of dye molecules across the gate (Fig. 2A, images). As expected, when the gate was lifted up, the fluorescent solution was pushed away from the gate, indicating a stream flowing from the water vessel to the dye vessel. The

fluorescence intensity measurement verified that this flow is effective to flush the dye solution away rapidly (Fig. 2A, bar graph). This hydrostatic pressure-driven flow was further studied in a μ COVE chip filled with 20- μ L aqueous suspension of fluorescent microparticles and 10- μ L water. Using a high-speed sCMOS camera, we observed that fluorescence microparticles traveled from the gate into the water vessel within ~ 120 ms after opening the gate (Fig. 2B). The averaged velocity of the initial flow caused by the hydrostatic pressure was estimated to be 7.8 ± 1.7 mm/s by tracking the microparticles ($n > 50$) within a timeframe of ~ 120 ms. The flow rate declined over time, as the solution flows from the particle vessel into the water vessel, diminishing the difference in the liquid levels in two vessels. The solutions in the two communicating vessels in our device was seen to approach approximately equal levels in one minute. Such rapid hydrodynamic counter flow is essential to reducing the carryover and non-specific binding on the microbeads without extended incubation for bead washing.

We then demonstrated magnetic bead transfer under the hydrodynamic counter flow between a vessel loaded with 1×10^6 magnetic beads in 10 μ L red food dye and the adjacent vessel filled with 20 μ L water. As seen in Fig. 2C, using a magnet, microbeads were concentrated at the closed gate and then pulled into the water vessel against the stream flowing into the dye-filled vessel after the valve was opened. No discernable loss of magnetic beads was observed after pulling through the gate under the counter flow and the hydrodynamic washing effectively removed the dye solution. The magnetic bead concentration and transfer process can be completed within one minute, which is compatible with other multiphase based methods^{29, 32, 34}. Bead recovery rate of the μ COVE chip was further determined by pulling different amounts of magnetic beads conjugated with FITC-labelled BSA through all seven vessels in the chip. By measuring the fluorescence before and after the assay sequence, a total bead loss of $\sim 2\%$ was consistently observed for the range of bead quantity that we typically used with the current chip design, i.e., from 5×10^5 to 1×10^6 beads (Fig. 2D). In contrast, multi-phase separation/washing requires a critical bead mass to overcome the surface tension, which determines the bead loss and is directly affected by the sample matrices and buffer composition^{32, 35}. A recently developed AirJump method based on the air/aqueous interface requires much more beads to achieve a total bead loss of less than 15%³² (Table 1).

μ COVE-based Immunoassays.

We sought to develop the μ COVE chip for biomarker detection by targeting human CEA and EGFR as the model targets. Overexpression of CEA and EGFR proteins has been widely used for clinical diagnosis and treatment of cancer, such as colorectal and lung cancer^{36, 37}. We first developed the μ COVE-based CEA ELISA in which the hydrodynamic washing method was used without extended incubation of magnetic beads in the washing buffer vessels (Fig. 1D). representative fluorescence images of the microreactor acquired after 30 min enzymatic signal amplification reaction are displayed in Fig. 3A, which shows a very low background level for the PBS blank and increasing fluorescence signal along with the CEA concentration. A logarithmic calibration curve was constructed by plotting the averaged fluorescence intensity in the microreactor as a function of CEA concentration (Fig. 3B). We demonstrated quantitative detection of CEA over an extensive dynamic range of

five orders of magnitude (3.2 pg/mL to 32 ng/mL). A low theoretical LOD of 1.0 pg/mL was determined by calculating the value of blank signal plus three standard deviations (S.D.), which compares comparably or favorably with the recently reported microfluidic methods^{38, 39} and biosensors^{40–43}, for sensitive detection of CEA protein. It is noted that for this pilot assay development stage, the fluorogenic signal amplification was performed with 0.2 mM RGP and 30 min incubation, which were selected from the reaction conditions commonly used in the literature^{44, 45} and can be further optimized to improve the assay sensitivity (see Fig. 3). An average of 7.1% relative standard deviation (RSD, $n = 3$) was obtained with multiple devices across the test concentration range, which is comparable with that reported for the commercial human CEA ELISA assays (e.g., <12% for the Invitrogen Kit; 4.4% for the Abcam SimpleStep Kit; and <12% for the BioVision Kit). To characterize the specificity of our assay, we detected CEA (3.2 ng/mL) in comparison to PBS blank containing 1% BSA and IgG (1 ng/mL) and EGFR (2 ng/mL) with anti-CEA mAb labelled magnetic beads, respectively. μ COVE-based ELISA detection of CEA with a low background measured from the blank, IgG and EGFR was demonstrated, as shown in Fig. 3C, which suggests the good specificity of the assay for CEA.

To further assess the μ COVE chip for different analyte targets, we attempted to establish an immunoassay for EGFR. The assay performance was optimized by fine tuning the assay protocol for CEA, especially the enzymatic reaction for fluorescence readout. It was observed that increasing the RGP concentration from 0.2 mM to 0.5 mM led to only a slight higher non-specific background but significant enhancement in signals (Fig. 4A). Higher RGP concentrations were seen to result in much higher background levels, which may in part attributed to the faster hydrolysis rate. To determine the optimal reaction time with 0.5 mM RGP, we studied the real-time enzymatic depletion of RGP substrate for measuring EGFR at various concentrations. As displayed in Fig. 4B, a constant reaction rate over 60 min incubation was observed at the EGFR concentration of 20 pg/mL, while at 200 pg/mL a ~2.8-fold reaction rate was obtained within ~30 min and reduced afterwards, implying significant substrate depletion. Therefore, we chose to use the reaction time of 30 min in this work to afford a high sensitivity for quantitative detection of EGFR. Shorter reaction time permits EGFR quantification at higher levels, but will result in lower readout signal and thus decreased detection sensitivity. Titration experiments using the optimized assay conditions showed quantitative detection of EGFR with a calculated LOD of 0.082 pg/mL (Fig. 4C). Such LOD at the fg/mL level is comparable with that of some sensitive biosensors and microdevices, such as nanoparticle-based bio-bar codes assisted by PCR amplification⁴⁶, whispering-gallery microcavity⁴⁷, and microfluidic digital ELISA⁴⁸. The detection signal was found to level off rapidly when the concentration was increased above 2 ng/mL, indicating a dynamic range of 4 orders of magnitude for EGFR detection. Specific detection of EGFR was also validated by comparing with the measurements of PBS blank, IgG, and CEA with the anti-EGFR immunomagnetic beads (Fig. 4D). Collectively, these results for both CEA and EGFR assays demonstrate the effectiveness of the rapid hydrodynamic washing method implemented in our μ COVE device to remove the unbound agents and non-specific background to enhance the signal-to-noise ratio.

To demonstrate the direct applicability of the μ COVE-based ELISA to the analysis of complex biological samples, we employed it to quantify the expression level of EGFR in

crude A431 whole cell lysate. Human epidermoid carcinoma A431 cells overexpress EGFR and thus are often used as a positive control cell line for EGFR expression. BSA-coated magnetic beads were used as a negative control to assess non-specific background. For comparison, two washing methods were used: the rapid hydrodynamic washing and the 10-min washing method in which the beads were pulled into a washing vessel and incubated for 10 min with rotation. As seen in Fig. 5, measurements of EGFR in the A431 crude lysate samples diluted to 0.376 and 3.76 $\mu\text{g/mL}$ yielded the fairly consistent levels of non-specific background and detection signals between the two washing methods. Additional 10-min washing reduced the background but also led to lower detection signal. This is mainly owing to enhanced dissociation of antibody-captured targets and the loss of beads during washing. Two washing methods resulted in the comparable signal levels after background correction. The signal-to-background ratios for the hydrodynamic washing vs. the 10-min washing were calculated to be 6.41 ± 1.17 vs. 6.77 ± 1.21 (0.376 $\mu\text{g/mL}$ lysate) and 7.08 ± 1.32 vs. 6.86 ± 0.85 (3.76 $\mu\text{g/mL}$ lysate), respectively, which are not significantly different between the two methods as assessed by the Student's *t* test. Applying the standard curve established immediately prior to the lysate measurements (Fig. 4C), the average EGFR level in the original A431 cell lysate measured with the 0.376 and 3.76 $\mu\text{g/mL}$ dilutions was calculated to be 326 ng/mL and 377 ng/mL, respectively, which closely matched with each other. These results further validate the effectiveness of the rapid hydrodynamic washing for the μCOVE -based immunoassay, enabling rapid and quantitative analysis of proteins in complex biological matrices. The whole assay was completed in 4 h, including an excessive 2-h incubation for protein capture to ensure high sensitivity. The assay time can be further shortened to ~ 2 h for detection of relative abundant targets. This turnaround time is much shorter than conventional ELISA, which normally takes 6 h to overnight,¹⁹ and comparable to some point-of-care ELISA chips reported.⁴⁹

Conclusion

In summary, we have developed a pneumatically gated μCOVE chip which employs the communicating vessel principle as a simple means to generate fast transient hydrodynamic flow for effective washing without the need of excessive incubation. Compared to traditional microfluidic immunoassays, our method greatly shortens and simplifies the washing steps to expedite the assay workflow. In contrast to the stationary multi-phase microfluidics developed for fast immuno-bead washing (Table 1), the single-phase, pneumatically gated μCOVE chip not only facilitates the transfer of magnetic beads to streamline the assay workflow, but also overcomes the limitations associated with the multi-phase operation. Our proof-of-concept studies demonstrated that the μCOVE device can afford highly sensitive and quantitative detection of two protein biomarkers, CEA and EGFR with the LOD as low as the sub-picogram per mL level. Direct detection of the expression of EGFR in the crude A431 cell lysate was also investigated to further validate the device for fast washing to expedite the multi-step immunomagnetic assay, enabling rapid and quantitative protein analysis with complex biological matrices. Overall, our work should present a novel microfluidic platform that combines the merits of the stationary multi-phase microfluidics and the flow-based microfluidics. The simplicity and high analytical performance of this

system would make it a useful platform for a broad range of applications in biological research and clinical diagnosis.

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Figure 1. Microfluidic communicating vessel (μCOVE) chip for rapid magnetic bead-based ELISA.

(A) Schematic illustration of the chip design. The three-layer PDMS/glass chip integrates four parallel units of gated communicating vessels, each consisting of seven vessels (~20 μL each) connected by the flow channels (red) in the bottom PDMS layer. The vessels are gated with an array of normally-closed microvalves actuated by the pneumatic channels (green) in the top layer. The vessel array is ended with an enclosed detection microchamber connected to a reservoir for loading RGP. (B) Photograph of an assembled chip filled with red food dye in the communicating vessels and green dye in the pneumatic control channels. (C) Assay workflow. Immunomagnetic microbeads are added into the sample vessel for target capture. After incubation, the first two gates are pneumatically opened one at a time to pull the beads across the next washing buffer vessel with a magnet. The beads are sequentially moved through the vessels containing biotinylated detection antibody, washing buffer, and streptavidin-β-galactosidase to form the immunocomplexes. Finally, the beads are pulled across two washing vessels into the detection microchamber loaded with RGP from the end reservoir. The tagged enzyme converts RGP to fluorescent resorufin molecules for quantitative measurement of the bead-captured proteins. (D) Schematic illustration of rapid hydrodynamic washing in a μCOVE chip. The hydrostatic pressure caused by the different liquid levels between two communicating vessels generates a counter flow to wash the beads during traverse. The washed beads are moved across the washing vessels without incubation to expedite the immunoassay. The drawing is not to scale.

Figure 2. Characterization of μCOVE for rapid flow washing of magnetic beads.

(A) Bright-field and fluorescence (left) microscopy images of the flow caused by the hydrostatic pressure in a μCOVE chip. The white lines indicate the microchannels and the gate that disconnects the left vessel filled with 10 μL of resorufin solution from the right vessel with 20 μL of water. When the gate was opened, the fluorescent solution was seen to be pushed away from the gate, indicating a stream flowing from the water vessel to the dye vessel. The fluorescence intensity at the indicated locations (a-c) was measured and plotted as the bar graph on the right. (B) Representative time-lapse images showing the flow of fluorescent microparticles (2 μm) after the gate was opened. The vessels were filled with 10 μL of water (left) and 20 μL of aqueous solution of microparticles (right), respectively. The averaged flow velocity was measured to be 7.8 ± 1.7 mm/s by tracking the trajectories of particles ($n > 50$). (C) Bright-field microscopy images showing magnetic beads being concentrated at the closed gate (top) and pulled through the opened gate (bottom) by a magnet against the water stream flowing into the dye solution vessel (white arrow). (D) Recovery rates of the magnetic beads pulled across all the vessels at different initial bead numbers.

Figure 3. Detection of carcinoembryonic antigen (CEA) using μ COVE chips.

(A) Representative fluorescence images (false color) and (B) log-log calibration curve of detecting a serial dilutions of CEA protein. Error bars represent one standard deviation (S.D., $n = 3$) obtained from different chips. The linear plot was obtained by the least-squares fitting. Dashed line indicates the background plus three times of the S.D. measured with the PBS blank. (C) Specificity evaluation of the CEA assay by measuring PBS blank, IgG (1 ng/mL), EGFR (2 ng/mL), and CEA (3.2 ng/mL), respectively. Error bars indicate one S.D. ($n =$

3). Enzymatic detection conditions for (A-C): [RGP] = 0.2 mM; incubation time = 30 min.
FL: fluorescence; A.U.: arbitrary unit.

Figure 4. Development of μ COVE-based EGFR ELISA.

(A) Effects of the RGP substrate concentration on enzymatic detection of EGFR. Error bars represent one S.D. ($n = 3$). (B) Time-lapse measurements of β -gal catalyzed fluorogenic reaction for detection of EGFR at 20 and 200 pg/mL, respectively. 0.5 mM RGP was used in this case. (C) Calibration curve of detecting a serial dilutions of EGFR standard. Error bars represent one S.D. ($n = 3$) from different chips. The linear plot was obtained by the least-squares fitting. Dashed line indicates the background plus three times the S.D. measured with the PBS blank. (D) Specificity evaluation of the EGFR assay by measuring PBS blank, IgG (1 ng/mL), CEA (3.2 ng/mL), and EGFR (2 ng/mL), respectively. Error bars indicate one S.D. ($n = 3$).

Figure 5. Detection of EGFR in A431 cell lysate.

The lysate (3.76 mg/mL) was prepared from human epidermoid carcinoma A431 cells overexpressing EGFR and diluted in PBSW solution by 1000 and 10000 times. The diluted cell lysate samples were assayed using magnetic beads coated with BSA and anti-EGFR antibody, respectively. The detection performance with the hydrodynamic washing was compared to that obtained by the 10-min washing method where beads were washing for additional 10 min in each washing vessel. Error bars represent one S.D. ($n = 3$).

Table 1.

Comparison of different immunoassay platforms

	96-well Plate ELISA	IFAST ³⁰	AirJump ³²	SlipChip ³³	μCOVE (this work)
Multi-phase System	No	Oil/Aqueous	Air/Aqueous	Oil/Aqueous	No
Separation/Washing Approach	Mechanical agitation	Volume pinning by surface tension	Volume pinning by surface tension	Serial dilution	Hydrodynamic counter flow
Wash Time per Step	~10 min (3×)	<1 min bead aggregating & pulling	1–2 min bead aggregating & pulling	Slow (4 cycles of diffusion & bead mixing)	< 1 min bead aggregating & pulling
Minimal Bead Amount Tested	N/A	~3 × 10 ⁶	~9 × 10 ⁶	N/A	5 × 10 ⁵
Bead Loss	N/A	Bead loss during traverse	15%	3%	2%
Lowest Concentration Detected	20 pg/mL EGFR	20 pg/mL PSA	N/A (only for sample prep.)	400 pg/mL insulin (LOD: 76 pg/mL)	0.2 pg/mL EGFR (LOD: 0.082 pg/mL); 3.2 pg/mL CEA (LOD: 1 pg/mL)