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Streamlining Immunoassays with Immiscible Filtrations Assisted by Surface Tension

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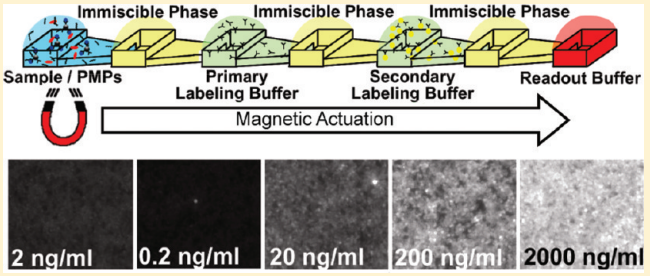
ABSTRACT: Immunoassays are utilized for a wide variety of clinical and biomedical research applications. In typical immunoassays, analytes are captured, labeled, and quantified on a single surface (e.g., the bottom of a well plate). In order to minimize the background, this type of assay must be washed multiple times between each of these steps to ensure residual reagents (e.g., unbound labeling antibody) are removed from the system. In this manuscript, the immunoassay is fundamentally reconfigured, such that each reagent is confined to its own well and no wash steps are required. Using immiscible filtration assisted by surface tension (IFAST), a technique developed for nucleic acid and whole cell purifications, immunoassays can be drastically simplified such that all reagent manipulation is performed at the start of the assay (i.e., no pipetting steps are necessary during the assay). Analytes are bound to paramagnetic particles via antibodies and drawn through oil barriers between four isolated compartments: (1) sample well, (2) primary antibody labeling well, (3) secondary antibody labeling well, and (4) readout buffer well. Using this technique, we have demonstrated repeatable detection of as little as 188 fg of protein. IFAST immunoassay functionality is demonstrated by detecting a well accepted prostate cancer biomarker, prostate specific antigen (PSA). Assay performance was assessed by measuring known concentrations of recombinant PSA protein. The assay was then used to measure PSA concentrations in conditioned media and human plasma samples.



The accurate and sensitive quantification of a biomolecular analyte from a sample is a requisite capability in many fields within the life sciences including medicine, pharmacology, and biomedical research. In particular, the discoveries of several biomarkers over the past few decades[1] have enabled doctors to draw conclusions about disease diagnosis, prognosis, and treatment. Biomarkers are biological molecules, often proteins, which correlate with a particular condition or disease, such as a specific type of cancer.[2] Quantification of the concentration of a biomarker from a patient sample, such as serum or urine, is often performed with an immunoassay, in which antibodies (Ab) are employed to specifically select for target analytes. While a few biomarkers have been in use for more than a decade, such as the prostate-specific antigen (PSA) test to detect prostate cancer (FDA approved in 1994[2]), new biomarkers are emerging at an accelerating pace.[3] Thus, improvements in immunoassay technology, specifically those that enable laboratories to perform facile testing with limited resources,[1] are necessary to ensure sufficient infrastructure to handle increased biomarker development and measurement.

Several immunoassay formats are currently available including enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescent immunoassay (FIA), each utilizing a different detectable molecule to label a target analyte and generate a signal proportional to analyte concentration. Typically, immunoassays are performed in microtiter plates (e.g., 96-well or 384-well plates) by sequentially adding and removing reagents from each well of the assay. These assays generally involve capturing a target analyte, either through Ab capture or adsorption, then labeling the captured analyte using a single or series of Abs linked to a molecule capable of generating readout (e.g., a fluorophore, an enzyme that generates a colored metabolite, or an analyte that is prelabeled with radioactive material). Since the entire assay typically takes place in a single compartment, multiple washing steps are commonly required between steps to ensure complete removal of unbound materials and nonspecific interacting molecules.

Standard laboratory protocols, including immunoassays, can be substantially simplified and expedited by leveraging new technology to eliminate or accelerate processing steps. One strategy involves binding analyte on a paramagnetic particle (PMP) and using a magnetic field to transport PMP-captured analyte between discrete compartments of the various reagents required by the assay. This type of compartmentalization has been achieved by separating droplets[4−7] or reservoirs[8−12] of aqueous reagents by an immiscible phase liquid (e.g., oil, wax), thus enabling isolation of nucleic acids,[4,5,9−11] protein-labeled DNA,[8] biotinylated enzyme,[6] or whole cells[12] in a streamlined format that requires little or no washing. Furthermore, since each step of the reaction is isolated from the others, reagents can be preloaded into the assay, thereby simplifying operation to the manipulation of the magnetic field required to transfer the PMP-bound material; no liquid manipulation is required once initial assay loading is complete. The elimination of

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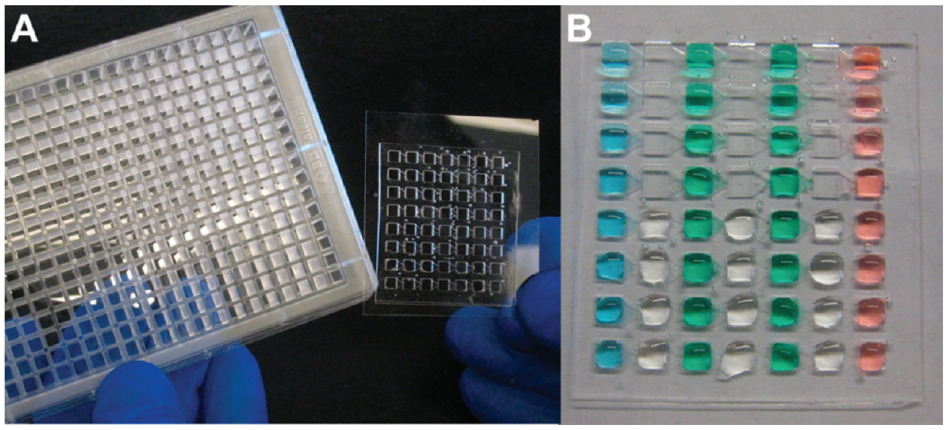

Figure 1. (A) Comparison of standard 384-well microtiter plate (BD Falcon) with an IFAST FIA array containing 8 assays (each row is an independent assay); (B) surface tension-mediated loading of the assay is demonstrated as the top four assays (rows) have been loaded only with the aqueous reagents (colored liquids) while the bottom four assays have been loaded with both the aqueous reagents and oil. Note that the aqueous reagents in the top four assays are pinned in place via surface tension despite the presence of adjacent empty wells.

washing during the immunoassay protocol represents a significant advantage, as these wash steps are time-consuming and a source for unwanted variability in the assay (e.g., contamination from residual wash buffer, dissociation of analyte during wash).

Here, we exploit the advantages of immiscible phase compartmentalization to develop an arrayed FIA platform to detect a diagnostically relevant analyte from a variety of different sample matrixes. This assay is very easy to operate both as a single unit and in arrays without sacrificing sensitivity. In order to facilitate adaptation by the life sciences community, the assay array is configured to mimic the footprint and appearance of a 384-well microtiter plate (Figure 1A), thus making it familiar and compatible with existing laboratory infrastructure (e.g., multichannel pipettes, liquid handling robots). The primary structural difference between our platform and a well plate is the existence of microscale conduits connecting a series of adjacent wells (seven wells form a single assay unit). These constricted conduits allow us to utilize the concept of "pinning"[8,10,13−15] to sequester a volume of liquid in a single well even though it is connected to its neighbor via the conduit. This phenomenon occurs because the fluid mechanics in this microscale region are governed by surface tension rather than gravity, as defined by a Bond number of much less than 1 ($Bo = \rho g L^2/\gamma$), where ρ is the liquid density, g is gravitational acceleration, γ is the liquid surface tension, and L is a characteristic length scale, taken to be the hydraulic diameter of the conduit.[8,10] Thus, the flow of a reagent through the conduit and into the adjacent chamber is associated with a large increase of surface tension/energy and is, therefore, energetically unfavorable. This concept is important because it allows the platform to be planar, with aqueous and oil wells side-by-side, as opposed to a configuration with entirely macroscale (high Bo) features, in which aqueous and oil phases stratify based upon density.[9] We term this planar configuration immiscible filtration assisted by surface tension or IFAST.

We validate our platform using a model system involving the detection of PSA, which, although somewhat controversial, is still the most common biomarker currently used to assist in the diagnosis of prostate cancer (PCa).[2] We demonstrate the versatility of our platform by quantifying PSA from a variety of sample matrixes including solution spiked with recombinant protein (for validation), human plasma, and conditioned media from PSA-secreting cells. This selection of matrixes highlights the broad potential impact of this platform by demonstrating utility in the research (biomarker development) and clinical (biomarker quantification) settings. Furthermore, applying our technology to streamline the immunoassay protocol will potentially accommodate the expanding biomarker quantification workload associated with the advent of new biomarkers. The compartmentalized nature of the IFAST platform facilitates easy integration with other components since a PMP-bound analyte can easily be transferred from one component to the next. We demonstrate this principle by integrating an arrayed IFAST FIA with cell culture microchannels. This configuration enables parallel quantification of secretions of multiple live cell cultures using only magnetic actuation to extract the secretions and quantify via FIA. This integration further streamlines the measurement process while mitigating the error associated with manually transferring the analyte from the culture platform to the IA platform.

MATERIALS AND METHODS

IFAST Design Fabrication. The IFAST FIA was designed to mimic the footprint of a standard 384-well microtiter plate in order to facilitate compatibility with existing laboratory infrastructure (e.g., multichannel pipets, liquid handling robots, plate readers) and to increase familiarity for potential users in the biomedical sciences. The devices were fabricated from poly(dimethyl siloxane) (PDMS) using soft lithographic techniques. Briefly, layers of a UV-curable epoxy (SU-8 100, Microchem) were spun onto a silicon wafer and patterned by using a mask to selectively expose the epoxy to UV light. Uncured epoxy was removed by washing with ethylene glycol followed by rinsing with isopropyl alcohol to complete formation of the epoxy mold. Uncured PDMS was poured on the mold and cured for 4 h at 85 °C. Cured PDMS was peeled off the mold and bonded to glass (Cover Glass, Thermo Scientific) using an oxygen plasma to enhance bonding. Each assay consists of seven wells oriented in a line, where the well-to-spacing is 4.5 mm. Since soft lithography, a planar process, was employed, the well height was only 750 μm, significantly shorter than a microtiter plate, resulting in a well volume of ~10 μL. Unlike microtiter plates, the wells are connected in sequence by a series of microfluidic constrictions (constriction area = 0.5−3.5 mm^2). These constrictions are small enough to

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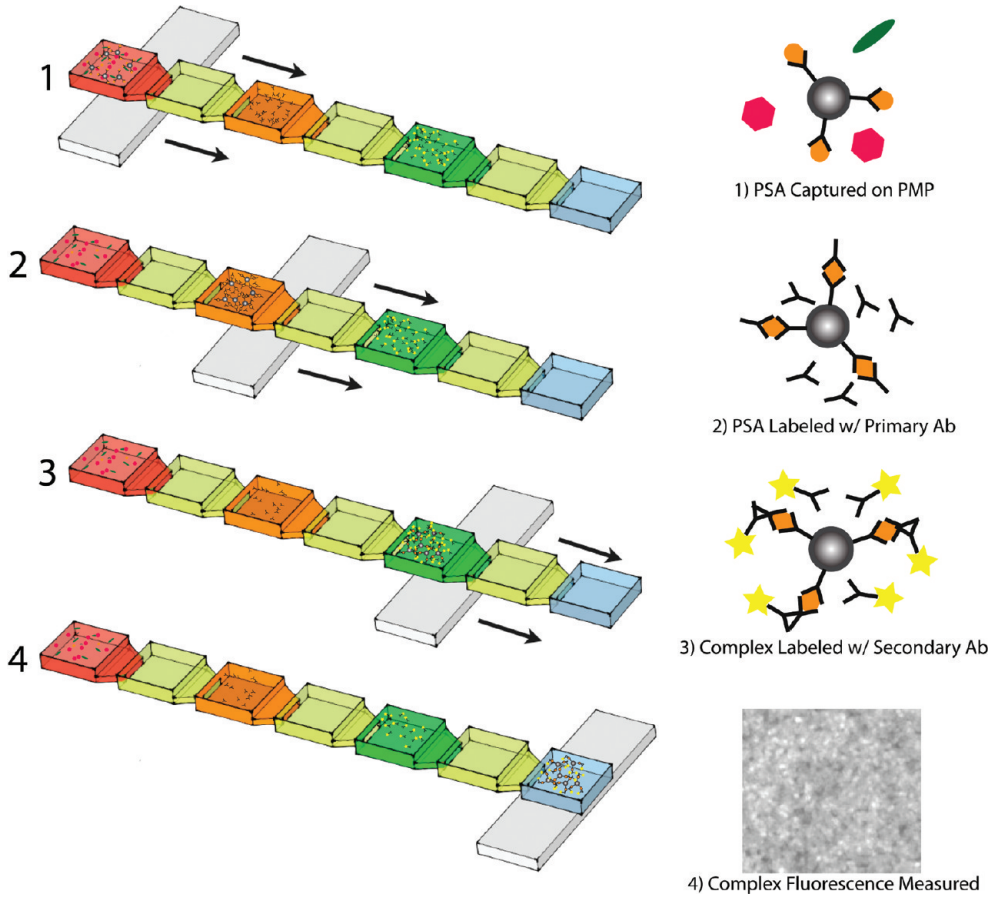

Figure 2. The device is loaded with the reagents and immiscible liquids as shown in Figure 1. A magnet draws the PMP-captured analyte through the first oil phase (1). The primary Ab binds with the analyte (2), and the magnet draws the analyte complex into the second oil phase, leaving the unbound primary Ab behind. The fluorescently labeled secondary Ab binds to the analyte complex via the primary Ab (3). The magnet is drawn through the final oil phase to separate the fluorescently labeled complex from the unbound secondary Ab, and the fluorescently labeled complex is imaged to quantify the analyte concentration (4).

facilitate liquid pinning, preventing liquid from exiting its well during filling, while allowing PMPs (and material captured on the PMP surface) the ability to move from well to well in response to a magnetic field.

PMP Preparation. For each assay, 5 μL of a 10 mg/mL stock solution of streptavadin-coated PMPs (Dynabeads M-280 Streptavadin, Invitrogen) were transferred to a 1.5 mL microcentrifuge tube and magnetically captured against the sidewall using a hand-held magnet (K&J Magnetics). The supernatant was aspirated, and the PMPs were washed once with 0.01% Tween 20 in PBS (PBST). After washing, the PMPs were resuspended in 10 μL of PBST, and 100 ng of biotinylated anti-PSA antibody (Ab) was added (ab77310, Abcam). After incubating for 30 min at room temperature, the PMPs were magnetically captured and the supernatant was removed. The PMPs were resuspended in 10 μL of PBST containing 5% goat serum (by volume) and incubated for 30 min at room temperature to block against nonspecific binding. Following incubation, the PMPs were resuspended in 10 μL of PBST and either used immediately or stored at +4 °C. While the volumes given here are per assay, PMPs were typically prepared in larger batches (10−50 assays) by scaling the volumes provided.

Sample Preparation. A variety of samples were incubated for 30 min at room temperature with PMP functionalized with capture Ab prepared as described in the previous paragraph to promote capture of sample PSA. For system characterization, various concentrations (20 pg/mL to 2 μg/mL) of recombinant PSA (ab78528, Abcam) in 1% BSA were used. To investigate the ability of the IFAST FIA to measure secreted PSA, PMPs were incubated with conditioned media from culture with PC-3 or LNCaP cells at time points of 4, 6, 8, 12, and 24 h. As a control, nonconditioned media (RPMI 1640 with 10% fetal bovine serum (FBS) for PC-3 cells; RPMI 1640 with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 7.5% sodium bicarbonate, and 2.6 g/L D-glucose for LNCaP cells) was also directly assayed (0 h time point). Human blood samples were collected from patients following IRB-approved protocols. Plasma was separated from whole blood using a Ficoll gradient, and plasma was stored at −80 °C until the FIA was run.

IFAST FIA Loading. Following incubation with PMPs functionalized with capture Ab, 8.5 μL of sample (recombinant PSA, conditioned media, or plasma) mixed with PMPs was added to the first well of the IFAST FIA. A volume of 8.5 μL of primary anti-PSA Ab (ab9537, Abcam) at a concentration of 1 μg/mL in 1% BSA was added to the third well while 8.5 μL of secondary antimouse Ab conjugated to an Alexa 647 fluorophore (Invitrogen) at a concentration of 200 ng/mL in 1% BSA was added to the fifth well. The seventh well was loaded with PBST, which acts as a readout buffer. Olive oil (Unilever) was added to the second, fourth, and sixth wells to complete the loading process (Figure 1B).

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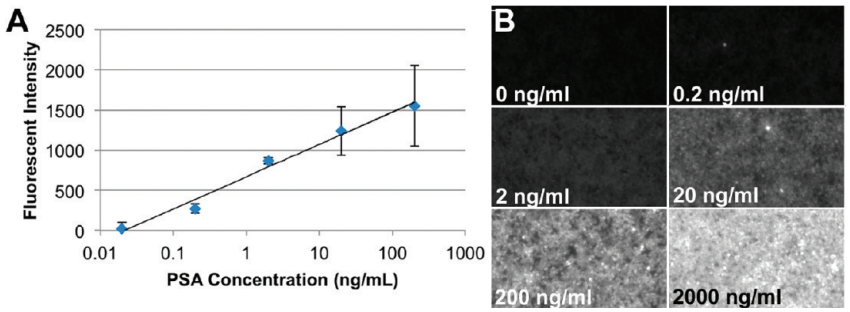

Figure 3. (A) Standard curve of fluorescent intensity as a function of recombinant PSA concentration (average % RSD = 20%, error bars represent standard deviation, n = 3, R^2 of semilog curve fit is 0.99) and (B) representative images of the fluorescent PMP/PSA complexes at the end of the assay.

IFAST FIA Operation. Once loaded, an array of eight IFAST FIA devices was placed directly on top of a long magnetic bar (BX041, K&J Magnetics) and moved such that the PMPs were drawn from the first well, through the oil in the second well, and into the primary Ab solution in the third well, thereby separating the captured PSA from the remainder of the sample. When in the oil phase, the hydrophilic PMPs (and associated analyte) formed a tight cluster, thus excluding the vast majority of unbound aqueous material. Additionally, the relatively high dipole moment of this paramagnetic cluster ensured that the PMPs were easy to move through the oil with a minimum of PMP loss during traverse. The magnet, which was moved manually in this investigation, was of sufficient length to actuate all eight devices simultaneously in parallel. The assay array was then removed from the magnet, and the PMPs were allowed to incubate in the primary Ab solution at room temperature for 1 h, during which time the assay was placed in an enclosed container (Omnitray, Nunc) in order to minimize evaporative loss. After primary incubation, the magnetic bar was utilized to draw the PMPs, now with primary Ab-labeled PSA, through the oil in the fourth well and into the secondary Ab solution in the fifth well. Again, the magnet was removed and the assay was incubated for 1 h at room temperature to facilitate attachment of the secondary Ab to the PMP/PSA/primary Ab complex. Following this incubation, the PMPs were drawn through the final oil phase and into the last well, thus separating bound material from unbound secondary Ab (Figure 2). In some cases, a micropipet was used to disperse the PMPs in each Ab well and in the readout well to promote more even binding and quantification.

Readout and Analysis. The final wells containing the PMP/PSA/Ab complex were imaged using a fluorescent microscope (IX70, Olympus) with a 10× objective and CCD camera (Hamamatsu). The wells were excited with light at 647 nm, and an image of the fluorescence was recorded. The average intensity was calculated using ImageJ software, and the background intensity was removed by subtracting the average intensity of a blank assay, which contained no Ab. Standard curves were constructed by plotting the fluorescence intensity as a function of recombinant PSA concentration. The fluorescent intensities of the assays were compared statistically using Student's t test.

RESULTS AND DISCUSSION

Assay Loading. Although all seven wells of the IFAST FIA are connected, each liquid was sequestered in a single well during device filling. The constrictions at the connection points between adjacent wells were sized such that the Bond number is sufficiently low at these locations (Bo < 0.01), indicating that surface tension will have substantial influence on the fluid mechanics at these interfaces. Since flow through a constriction and into an adjacent well is associated with an increase in surface area (and associated surface energy), flow through the constriction is energetically unfavorable and the liquid becomes pinned at the edge of the constriction. This phenomenon allows immiscible liquids to be positioned side-by-side rather than stratified by density, as would occur if the constriction (and associated Bo) were too large. In summary, this principle facilitates the establishment of a series of liquid volumes that are both connected and discrete, enabling a PMP-bound analyte to contact a series of reagents without any manipulation of liquid beyond initial reagent loading. This methodology provides an alternative to the typical microtiter plate paradigm, in which reagents are sequentially aspirated from and loaded into a single well, often with washing steps between functional reagents to remove residual components (e.g., unbound antibodies or fluorophores) that may erroneously affect downstream processes or measurements.

Assay Characterization. To characterize the performance of the IFAST FIA, the assay was performed using a series of dilutions of recombinant PSA with known concentrations. It was found that the fluorescent intensity of the PMPs in the final well was monotonically correlated to the PSA concentration. After background subtraction (the intensity of the blank measurement was subtracted from the nonblank data points), this relationship is approximated by fitting an exponential function:

$$C_{PSA} = e^{(I_{FL} - a/b)} \quad (1)$$

where C_{PSA} is the PSA concentration (in ng/mL), I_{FL} is the fluorescent intensity (Figure 3A), a is an offset constant (equal to 688.1 based on the standard curve in Figure 3A), and b is a scaling constant (equal to 112.3 in this instance, as per standard curve). As with many immunoassays, we recommend constructing a standard curve with each set of experiments using samples with known concentrations in order to correct any assay bias, which could lead to inaccurate results. Representative images of the PMPs following completion of the assay with known PSA concentrations are shown in Figure 3B–E. These data suggest that the IFAST FIA can successfully detect PSA concentrations as low as 20 pg/mL, with repeatable detection at 200 pg/mL. Given the small size of the IFAST input well (8.5 μL), this concentration (200 pg/mL) represents only 188 fg of PSA. The relative standard deviation (% RSD) at this value was found to be 21% (n = 3) and an average of 20% over the tested range. While this level is higher than

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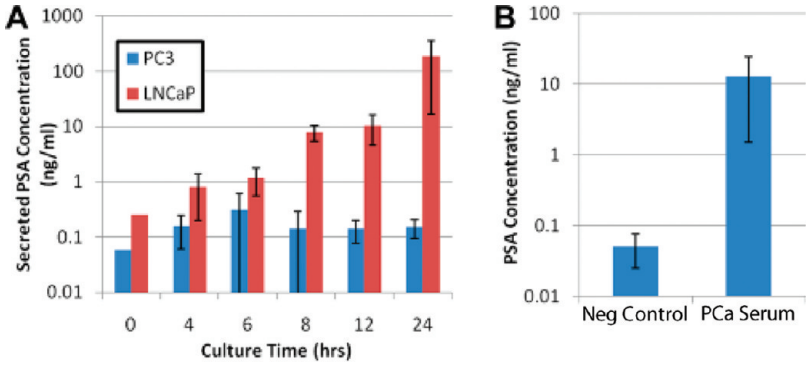

Figure 4. (A) Media conditioned with LNCaP cells for up to 24 h contains significantly more PSA than media conditioned with non-PSA secreting cells (PC-3 cells). Error bars represent standard deviation with three biological replicates; (B) serum from a PCa negative patient shows little to no PSA while serum from a prostate cancer patient exhibits an elevated PSA level. Samples were run three times, and error bars represent the standard deviation.

commercial assays (3−10% RSD reported for commercial PSA assays[16−18]), we believe that these values demonstrate reasonable precision for a "proof-of-concept" of a new immunoassay technology.

The upper limit of detection for this assay was found to be 2 μg/mL, representing 4−5 orders of magnitude with a single set of Ab concentrations. During preliminary experimentation, we noticed that this range could be extended upward by increasing the concentration of the primary and secondary Ab but that the background also increased, thus limiting our lower limit of detection. Since patient PSA concentrations are typically within a range extending from <1 ng/mL (healthy individual) to >1 μg/mL (advanced cancer),[19] we engineered the assay to be sensitive within this range. However, this range can likely be tuned for other applications requiring detection of other analyte concentrations via reoptimization of the Ab quantities. Furthermore, we found no statistically significant difference (p = 0.67) between trials where a micropipet was used to disperse the PMPs in each reagent well and trials where the PMPs were not manipulated in any way other than with the magnet. This result suggests that once the assays are loaded, no further liquid handling steps are necessary, thus promoting limited loss of sample by extremely facile operation of the platform.

Biological Sample Measurements. To demonstrate the direct applicability of the IFAST FIA for research and clinical applications, it was employed to detect the presence of PSA in conditioned media and human samples. A line of human epithelial cancer cells (LNCaP), which are known to express PSA,[20] were cultured in media for up to 24 h and then the media was collected and 8.5 μL aliquots were assayed using the IFAST FIA. Aliquots of media conditioned with PC-3 cells, which are human PCa cells known not to express PSA,[21] were used as a control. Assays of media conditioned with LNCaP cells resulted in a significant increase (p < 0.02) in PMP fluorescence relative to the unconditioned media controls for the 8, 12, and 24 h time points compared to the media conditioned with PC-3 cells. Using calculations based on the standard curve shown in Figure 3A, the average PSA level in the media conditioned with LNCaP cells rose to 185 ng/mL while the PSA levels in the PC-3 conditioned media remained around 100 pg/mL for all time points, which is below the limit of detection (200 pg/mL) of the IFAST FIA (Figure 4A). Similarly, assay signal was found to be significantly higher (p < 0.01) when probing PSA levels in a PCa patient compared to serum from a female patient (PCa negative control). Applying the standard curve, PSA levels in serum from a PCa patient were found to be much higher (13.0 ng/mL on average) than levels from the negative control patient (50 pg/mL on average), which was also below the limit of detection of the assay (Figure 4B).

■ CONCLUSIONS

A new, arrayed FIA assay has been developed and utilized to detect a diagnostically important protein, PSA, from a variety of sample types. Unlike conventional immunoassays, no washing or other liquid handling steps are necessary once the device has been loaded. Instead, PMP-captured analytes are transferred via a magnetic field between volumes of aqueous reagents separated by immiscible liquid barriers. This assay is configured like a microtiter plate, possessing a familiar, open well format that allows easy loading (and removal, if desired) of reagents. This simplistic array is enabled by the surface tension-dominant fluid mechanics associated with microscale conduit geometries to position aqueous and oil phases side by side. Characterization of the device illustrated sensitivity comparable to commercial immunoassays and a physiologically relevant detection range spanning 4−5 orders of magnitude.

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Notes

S.M. Berry and D.J. Beebe have an ownership interest in Elaion Biosciences, Inc.

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■ REFERENCES

(1) Vilanueva, J.; Shafer, D. R.; Philip, J.; Chaparro, C. C.; Erdjument-Bromage, H.; et al. *J. Clin. Invest.* **2006**, *116*, 271−284.
(2) Madu, C. O.; Lu, Y. *J. Cancer* **2010**, *1*, 150−177.
(3) Sidransky, D. *Nat. Rev.* **2002**, *2*, 210−219.
(4) Okochi, M.; Tsuchiya, H.; Kumazawa, F.; Shikida, M.; Honda, H. *J. Biosci. Bioeng.* **2010**, *109*, 193−197.

(5) Tsuchiya, H.; Okochi, M.; Nagao, N.; Shikida, M.; Honda, H. *Sens. Actuators, B* **2008**, *130*, 583−588.
(6) Shikida, M.; Takayanagi, K.; Honda, H.; Ito, H.; Sato, K. J. *J. Micromech. Microeng.* **2006**, *16*, 1875−1883.
(7) Shikida, M.; Takayanagi, K.; Inouchi, K.; Honda, H.; Sato, K. *Sens. Actuators, B* **2006**, *113*, 563−569.
(8) Chen, H.; Abolmatty, A.; Faghri, M. *Microfluid. Nanofluid.* **2010**, *10*, 593−605.
(9) Sur, K.; McFall, S. M.; Yeh, E. T.; Jangam, S. R.; Hayden, M. A.; Stroupe, S. D.; Kelso, D. M. *J. Mol. Diagn.* **2010**, *12*, 620−628.
(10) Berry, S. M.; Alarid, E. T.; Beebe, D. J. *Lab Chip* **2011**, *11*, 1747−1753.
(11) Bordelon, H.; Adams, N. M.; Klemm, A. S.; Russ, P. K.; Williams, J. V.; Talbot, H. K.; Wright, D. W.; Haselton, F. R. *ACS Appl. Mater. Interfaces* **2011**, *3* (6), 2161−2168.
(12) Berry, S. M.; Strotman, L. N.; Kueck, J. D.; Alarid, E. T.; Beebe, D. J. *Biomed. Microdevices* **2011**, *13*, 1033−42.
(13) Atencia, J.; Beebe, D. J. *Nature* **2005**, *437*, 648−655.
(14) Zhao, B.; Moore, J. S.; Beebe, D. J. *Science* **2001**, *291*, 1023−1026.
(15) Cho, H.; Kim, H.-Y.; Kang, J. Y.; Kim, T. S. *J. Colloid Interface Sci.* **2007**, *306*, 379−385.
(16) Abazyme LLC. Human PSA ELISA Kit Protocol, 2004.
(17) Quantikine. Human Kallikrein 3/PSA Immunoassay Protocol, 2010.
(18) Anogen. Human Free PSA ELISA Kit, 2002.
(19) Connolly, D. J.; Black, A.; Murray, L. J.; Gavin, A.; Keane, P. F. *Prostate Cancer Symposium*, February 22−24, 2007, Orlando, FL, USA.
(20) Webber, M. M.; Bello, D.; Quader, S. *Prostate* **1997**, *30*, 58−64.
(21) Van Bokhoven, A.; Varella-Garcia, M.; Korch, C.; Johannes, W. U.; Smith, E. E.; et al. *Prostate* **2003**, *57*, 205−225.