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ORIGINAL PAPER

A digital microfluidic approach to heterogeneous immunoassays

Elizabeth M. Miller · Alphonsus H. C. Ng · Uvaraj Uddayasankar · Aaron R. Wheeler

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Abstract A digital microfluidic (DMF) device was applied to a heterogeneous sandwich immunoassay. The digital approach to microfluidics manipulates samples and reagents in the form of discrete droplets, as opposed to the streams of fluid used in microchannels. Since droplets are manipulated on relatively generic 2-D arrays of electrodes, DMF devices are straightforward to use, and are reconfigurable for any desired combination of droplet operations. This flexibility makes them suitable for a wide range of applications, especially those requiring long, multistep protocols such as immunoassays. Here, we developed an immunoassay on a DMF device using Human IgG as a model analyte. To capture the analyte, an anti-IgG antibody was physisorbed on the hydrophobic surface of a DMF device, and DMF actuation was used for all washing and incubation steps. The bound analyte was detected using FITC-labeled anti-IgG, and fluorescence after the final wash was measured in a fluorescence plate reader. A non-ionic polymer surfactant, Pluronic F-127, was added to sample and detection antibody solutions to control non-specific binding and aid in movement via DMF. Sample and reagent volumes were reduced by nearly three orders of magnitude relative to conventional multiwell plate methods. Since droplets are in constant motion, the antibody–antigen binding kinetics is not limited by diffusion, and total analysis times were reduced to less than 2.5 h per assay. A multiplexed device comprising several DMF platforms wired in series further increased the throughput of the technique. A dynamic range of approximately one order of magnitude was achieved, with reproducibility similar to the assay when performed in a 96-well plate. In bovine serum samples spiked with human IgG, the target molecule was successfully detected in the presence of a 100-fold excess of bovine IgG. It was concluded that the digital microfluidic format is capable of carrying out qualitative and quantitative sandwich immunoassays with a dramatic reduction in reagent usage and analysis time compared to macroscale methods.

Keywords Digital microfluidics · Immunoassay · Electrowetting

E. M. Miller · U. Uddayasankar · A. R. Wheeler
Department of Chemistry, University of Toronto,
80 St George St.,
Toronto, ON M5S 3H6, Canada

A. H. C. Ng · A. R. Wheeler
Institute of Biomaterials and Biomedical Engineering,
164 College Street,
Toronto, ON M5S 3G9, Canada

E. M. Miller · A. H. C. Ng · U. Uddayasankar · A. R. Wheeler (✉)
Donnelly Centre for Cellular and Biomolecular Research,
160 College St.,
Toronto, ON M5S 3E1, Canada
e-mail: aaron.wheeler@utoronto.ca

Introduction

Immunoassays are a sensitive and powerful analytical tool, having been used for applications ranging from diagnosing pregnancy [1] and malaria [2] to detecting biological warfare agents.[3, 4] The very high specificity of the antibody–antigen interaction means that these tests have a low rate of false positives and can detect very low quantities of a target molecule in complex samples. An immunoassay can be placed into one of two broad categories: homogeneous, or solution-based assays, and heterogeneous, or surface-based assays. Homogeneous assays can have excellent sensitivity, but often require a

separation technique such as capillary electrophoresis to distinguish bound from unbound antibody. In contrast, heterogeneous assays do not require a separation step, as the bound antibody complex is confined to a surface and unbound antigen or antibodies are washed away. The surface in such assays can be permanent (e.g., the bottom of a well in a multiwell plate) or mobile (e.g., magnetic beads). A common type of heterogeneous immunoassay is a sandwich assay, in which a capture antibody is immobilized to a surface, which binds a target antigen when sample is introduced. The surface is washed and is exposed to a secondary antibody with a detectable (i.e., fluorescent or enzymatic) tag, and washed again before detection. Unfortunately, this process is time-consuming in conventional multiwell plate immunoassays, as each binding step is limited by the time required for molecules to diffuse to the surface. In addition, antibodies are expensive, as antibody production is an expensive and laborious process. As a result, immunoassays are an ideal application for microfluidic systems, in which smaller dimensions reduce diffusion times and reagent costs, resulting in faster analysis times and higher throughput.[5]

Here, we report the development of an immunoassay system relying on digital microfluidics (DMF), a fluid handling technique in which droplets are manipulated by applying electrical fields to an array of electrodes.[6] In contrast to the more conventional geometry of enclosed microchannels, in DMF, each sample and reagent droplet is individually addressable,[7] making it suitable for applications requiring long, multistep protocols. Using this technique, droplets can be driven to move, merge, split into smaller droplets, and dispense from reservoirs.[8] There is much enthusiasm for using DMF for applications such as cell culture and assays,[9, 10] DNA sample processing and analysis,[11–15] protein sample processing and analysis,[16–21] enzyme assays,[22–24] and clinical sample processing.[16] To date, two heterogeneous immunoassay methods based on digital microfluidics have been reported, both of which rely upon particles suspended in droplets. In the first approach,[25] assays were developed for IgG and ricin based on the agglutination of latex and gold particles. A droplet suspended in fluorinated oil on a single plate device served as a container with a controlled evaporation rate; the pattern assumed by the antibody-coated latex and gold particles as the droplet evaporated was indicative of the quantity of antigen present in the sample. This method reduced sample volumes and limits of detection compared to commercially available methods, but has a readout that may be difficult to standardize or automate for high-throughput analysis. In the second approach, magnetic beads were used as a support surface for sandwich assays using fluorescent or chemiluminescent detection.[26, 27] While the use of beads ensures high surface area for contact between the antibody-coated surface and the sample and takes advantage of the faster mass transport conditions in the microscale (relative to well plates), an external magnet is required to separate the beads from the supernatant for the requisite wash steps, complicating device design and droplet control.

An alternative to using beads as a solid support for heterogeneous immunoassays is direct immobilization of capture antibody on the surface of the device. Similar types of surface modification of DMF devices have previously been reported for adherent cell culture [9] and for detection of DNA hybridization via surface plasmon resonance (SPR) imaging,[11] but this strategy has yet to be explored for heterogeneous immunoassays. Here, we demonstrate the first application of digital microfluidics to the detection and quantification of proteins via a heterogeneous immunoassay with no need for beads or magnets, liquid suspending media (e.g, silicone oil), or bovine serum albumin (BSA) surface blocking. We show that the DMF assay has similar sensitivity and dynamic range to conventional methods, but with much faster processing times. This technique has great potential as a simple yet versatile analytical tool for implementing immunoassays on the microscale.

Experimental

Reagents and materials Anti-human IgG antibodies (F_c-specific, and fluorescein isothiocyanate (FITC)-labeled F_{ab}-specific, raised in goat), human IgG (reagent grade, from serum), BSA, bovine serum, Tween 20, sodium phosphate dibasic, and Pluronic F-127 were purchased from Sigma Chemical (Oakville, ON). All solutions (sample, rinse buffers, capture antibodies, detection antibodies, and diluted serum) were formed in deionized water (18 M Ω cm) and contained 0.00625% (*w*/*v*) Pluronic F-127 (except for diluted serum, which contained 0.0113% Pluronic F-127). Sample, antibody, and antigen solutions contained 10 mM phosphate buffer, pH 7.4. Parylene C dimer was from Specialty Coating Systems (Indianapolis, IN), and Cytop resin solution (CT-809M) and solvent (CT-SOLV180) were purchased from Bellex International (Wilmington, DE).

Device fabrication Glass substrates bearing patterned chromium electrodes (used as bottom plates of DMF devices) were formed by photolithography and etching as described previously [22] in the University of Toronto Emerging Communications Technology Institute fabrication facility. Two similar device designs were used, featuring assay zones comprising ten or 20 1 mm×1 mm actuation electrodes and two or four 2.5 mm×2.5 mm reservoir electrodes, with inter-electrode gaps of 40–75 μm. Each

device contained six or ten assay zones with homologous electrodes bussed together (Fig. 1) such that multiplexed assays could be carried out in parallel. After forming electrodes, the substrates were coated with 2 μm of parylene C and 130 nm of Cytop. Parylene C was applied using a vapor deposition instrument (Specialty Coating Systems), and Cytop was spin-coated (2.25% *w/w* in CT-SOLV180, 1,000 RPM, 60 s) and then post-baked on a hot plate (180 °C, 15 min). To actuate droplets, the polymer coatings were locally removed from the contact pads by gentle scraping with wafer tweezers. In addition to patterned devices, unpatterned indium tin oxide (ITO)-coated glass substrates (Delta Technologies Ltd, Stillwater, MN) were coated with Cytop (130 nm, as above) to serve as device top plates.

Droplet actuation Each device was assembled with an unpatterned ITO/glass top plate and a patterned bottom plate separated by a spacer formed from two pieces of double-sided tape (~150-μm thick). As described previously,[22] droplets were sandwiched between the two plates and actuated by applying electric potentials between the top electrode and sequential electrodes on the bottom plate (Fig. 1a). Driving potentials (75 V_{RMS}) were generated by amplifying the output of a function generator operating at 18 kHz and were applied manually to exposed contact pads on the bottom plate surface. Droplet actuation was monitored and recorded by a CCD camera mated to a stereomicroscope with fluorescence imaging capability (Olympus Canada, Markham, ON).

Immobilization of capture antibody Capture antibody (F_c-specific anti-human IgG) was immobilized to an unpatterned Cytop/ITO-coated glass slide by manual pipetting of 250–500 nL of a 0.22–2.2-mg/mL solution onto the slide. Spots were allowed to air-dry, then rinsed in a 0.1% (*w/v*) solution of Pluronic F-127 in deionized water and allowed to dry before use. These concentrations were selected to ensure saturation of the surface with antibody in the area of the spot.

Optimization of DMF assay protocol To characterize antibody–antigen binding in a DMF environment, a truncated assay (different from the full sandwich immunoassay, which is described below) was implemented, in which 500 nL of a 0.57 mg/mL human IgG was spotted on an ITO slide, rinsed, and dried as above. This concentration was chosen to ensure complete saturation of the printed spot with antigen. DMF actuation was used to move a droplet of FITC-anti-human IgG across the antigen spot, and a fluorescence measurement was taken after each pass of the detection antibody. To probe non-specific binding on the device, measurements were also collected from the native surface after each pass, and from spots of BSA, spotted as above, but containing no human IgG.

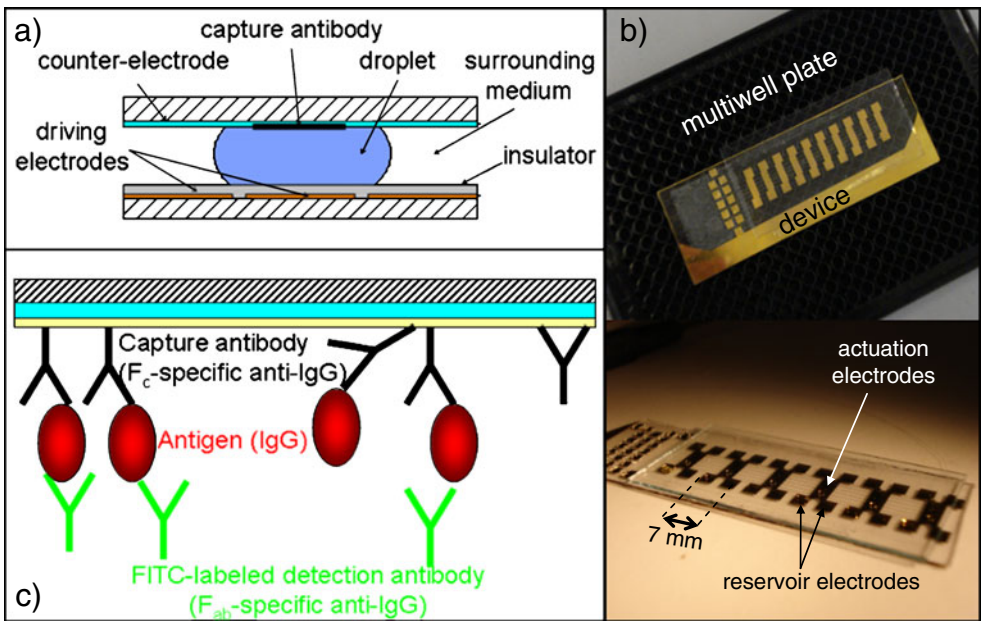

Fig. 1 A DMF device for heterogeneous immunoassays. (**a**) Schematic depicting the device. The driving electrodes are coated with an insulator and a layer of Cytop, while the indium tin oxide (ITO) counter electrode is coated with Cytop and bears a spot of printed capture antibody. The driving electrodes are actuated in series to move the droplet across the spotted antibody. (**b**) Pictures of 10- (*top*) and 6- (*bottom*) platform DMF devices. The actuation platforms are wired in series for simultaneous control of replicate assays, and the devices are placed on a multiwell plate for fluorescence measurements. (**c**) Schematic depicting an IgG sandwich immunoassay. The unpatterned cover plate is spotted with capture antibody (anti-human IgG), which binds the antigen (IgG) from the sample, which binds the detection antibody (FITC-labeled anti-human IgG)

Heterogeneous DMF immunoassay All steps of the sandwich immunoassay were implemented via DMF actuation on devices positioned on the top of a multiwell plate (Fig. 1). One-microliter aliquots of antigen sample (human IgG) and rinse buffer (10 mM phosphate buffer containing 0.00625% Pluronic F-127 *w/v*) were loaded into reservoirs on the DMF device before assembling with the pre-spotted top plate. As shown in Fig. 2, DMF actuation was used to move a 1-μL sample droplet to the capture antibody spot for antigen binding and to cycle it back and forth across the spot ten times. The sample droplet was then withdrawn to the reservoir, and a 1-μL droplet of rinse buffer was actuated across the spot to rinse away any non-specifically bound IgG. The waste droplet was then manually removed from the chip with a laboratory tissue, and the device was reloaded with 1-μL aliquots of detection antibody (FITC-F_{ab}-specific anti-human IgG) and rinse buffer. After replacing the Cytop/ITO cover plate, DMF was used to deliver the detection antibody solution to the assay spot, to cycle it back and forth ten times, to remove it, and to perform a final rinse step (Fig. 2).

After all assay steps were completed, the device (still positioned on top of the multiwell plate) was inserted into a PheraStar multiwell plate reader (BMG Labtech, Durham, NC) for fluorescence detection (λ_{ex}=485 nm; λ_{em}=520 nm; focal height=12.1 mm). After each assay, the Cytop/ITO

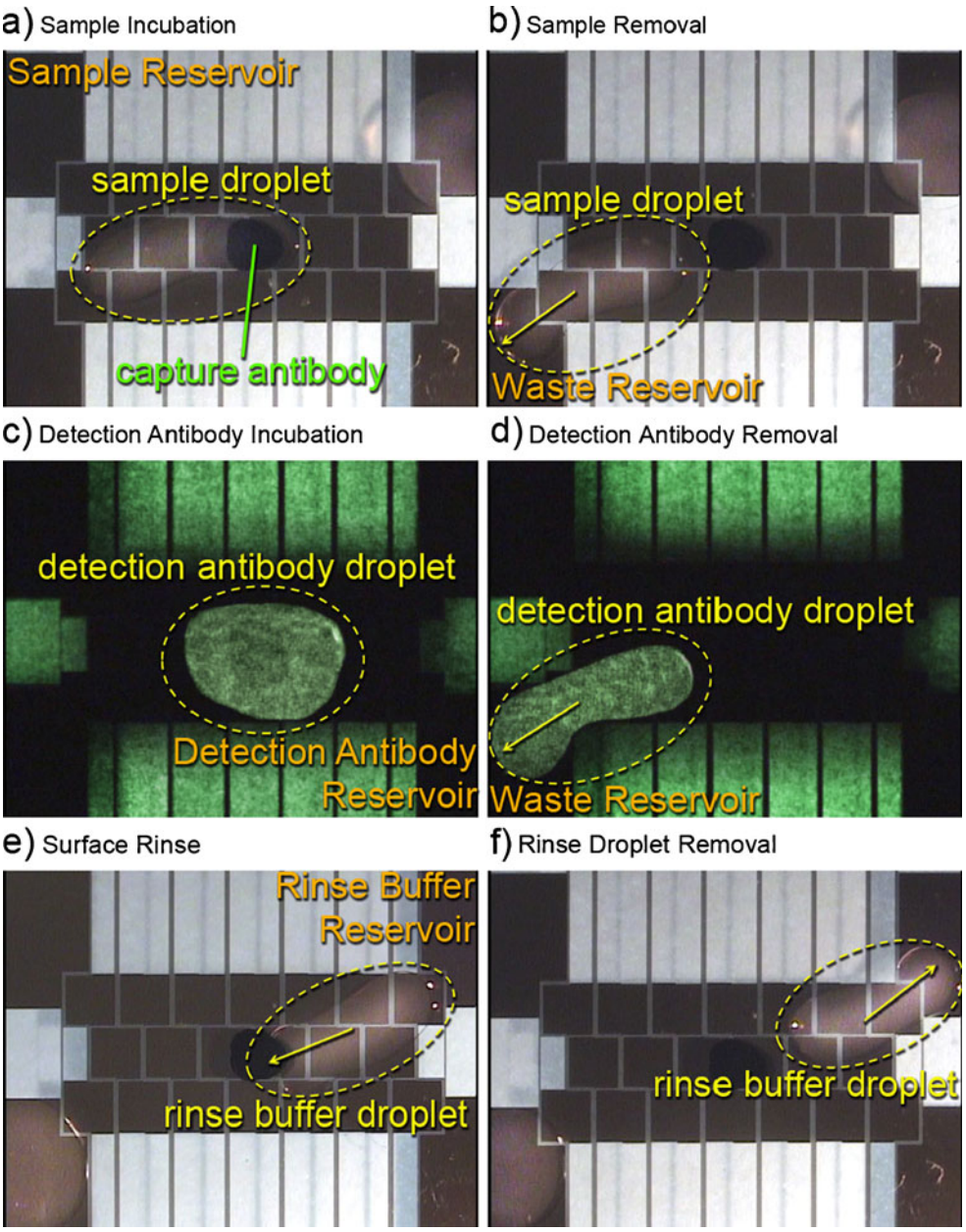

Fig. 2 Frames from a movie depicting a heterogeneous immunoassay on a DMF device. The *black spot* in the center of the device marks the location of the immobilized capture antibody (anti-IgG). **a** A droplet containing antigen (human IgG) is moved to the capture antibody via DMF actuation and **b** removed. A rinse cycle is performed, then a droplet containing detection antibody (FITC-labeled anti-IgG) is moved across the spot (**c**) and removed (**d**). A final rinse is performed (**e**) and the droplet is removed to a waste reservoir (**f**) before detection on the spot via a fluorescence plate reader. *Dotted lines* were added to aid observation of transparent droplets

cover plate was discarded, and the patterned bottom plate was re-used in subsequent experiments. For all experiments, three to five trials were conducted to evaluate reproducibility.

For comparison, the same assay performed on the DMF device (in 1-μL droplets, as described above) was also carried out in 96-well multiwell plates (in 100 μL aliquots) using a published commercial protocol,[28] with three to five replicates of each measurement. To ensure that both methods were implemented under comparable conditions, all experiments in multiwell plates used the same absolute mass amounts of antigen used on digital microfluidic devices.

DMF immunoassay of doped bovine serum Whole serum was diluted 100× and then spiked with human IgG (0–10 μg/mL final concentration), and the DMF assay was performed as described above.

Results and discussion

Device optimization In developing the new method reported here, we considered two different positions for the surface-bound antibodies: the unpatterned top plate or the patterned bottom plate. After some trial and error, we chose to use the top plate for three reasons: (1) the top plate is simpler to fabricate and thus easier to replace than the grid of actuation electrodes on the bottom plate; (2) the process of printing, drying, and rinsing the capture antibody spots can damage the insulator on the bottom plate, making it susceptible to electrolysis (as opposed to the top plate, which does not bear an insulating layer); and (3) the surface area of the capture antibody spot is approximately the same size as the actuation electrodes, but is small relative to the size of the ground electrode, and therefore is less likely to interfere with its role in actuation. In addition, by using the top plate for the sandwich assay, the lifetime of the bottom plate of the device (the time-consuming piece to fabricate) is extended such that it can be used for multiple assays before it must be discarded. We note that a DMF top plate was also recently used for surface immobilization of DNA for SPR measurements, [11] presumably for similar reasons.

A related challenge for this work was the ruggedness of the hydrophobic coating on the device surface. Typically, DMF devices are made hydrophobic by spin coating a 50-nm layer of Teflon-AF on the actuation and ground electrodes. In this work, Teflon-AF was found to be too delicate for implementing a surface-based assay via DMF; the process of printing the capture antibody generated flaws and tears in the Teflon surface, causing solutions to wet the surface and making DMF actuation impossible. Thus, in this work, a 130-nm Cytop coating was used, which proved to be more rugged, allowing spots to be dried and rinsed without causing the hydrophobic layer to separate from the electrode surface and collect fluid.

Optimization of assay conditions Aqueous solutions containing proteins are known to be problematic for digital microfluidics—when proteins adsorb to the device surfaces, the surfaces become hydrophilic, and resist droplet movement. One solution to this problem is to include non-ionic polymer surfactant additives in protein-containing solutions used on DMF systems to reduce the extent of protein adsorption.[22, 29] Here, we used Pluronic F-127, which was included as a constituent of all solutions as described in the Experimental section.

In some DMF device configurations, actuated droplets are suspended in an immiscible silicone oil to aid in droplet movement and prevent non-specific adsorption of proteins to the device surface.[23] In the system described here, the Pluronic serves the purpose of preventing non-specific adsorption, and carrier oil is not required. This is an essential distinction, as in other systems,[23] the carrier oil surrounds the entire droplet, forming a barrier between the droplet and the surface. This barrier is likely to interfere with antibody–antigen binding events at the surface, and could possibly render the assay ineffective. In the methods described here, droplets are surrounded only by air, allowing them to make contact with the assay surface.

DMF protocol development Since the digital microfluidic assay was performed in a dynamic system (i.e., with droplets moving) instead of a static environment (e.g., the contents of a well plate that is not being stirred or shaken), we expected that the binding kinetics during each step would be accelerated relative to those in bulk solution. In addition, because the sample solution spends less time in contact with the assay surface, non-specific adsorption should also be reduced relative to a static system. To test this hypothesis and to optimize the DMF actuation steps for sample binding relative to non-specific adsorption, a truncated assay was developed in which spots of the antigen (human IgG) were deposited directly on the ITO slide, and droplets of detection antibody were actuated across each spot and withdrawn to their respective reservoirs. A fluorescence measurement was taken after each actuation step to plot a binding curve as a function of the number of passes by the detection antibody (Fig. 3). The binding curve was reproducible (relative standard deviation (%RSD)<10% in each point), and it indicates that after only two passes of the droplet containing the detection antibody, the assay spot had bound as much antibody as possible from the droplet. Fluorescence measurements of the detection antibody droplets during

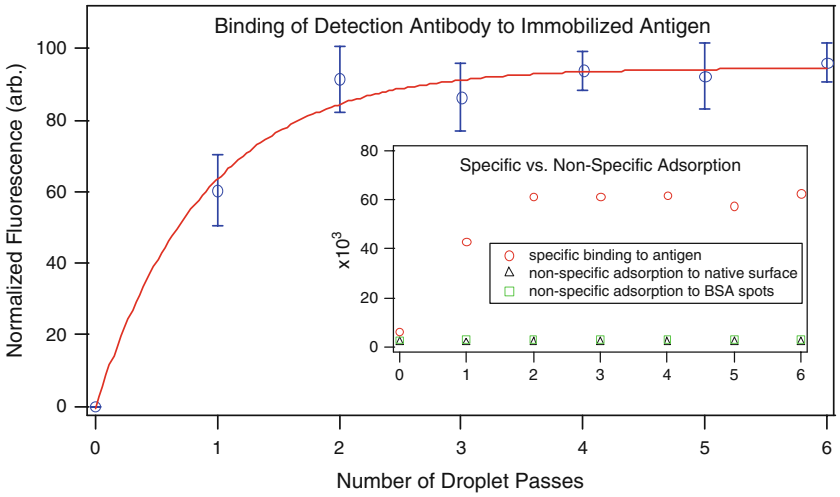

Fig. 3 Optimization of DMF protocol as a function of binding of FITC-labeled detection antibody to immobilized antigen. Antigen (IgG) spots were immobilized on the cover plate and droplets containing detection antibody were translated across the spot via DMF. Fluorescence measurements after each pass of the droplet indicate that after two passes, the spot has bound all available antibody from the droplet (*main panel*). Each *data point* represents five replicate trials and the *error bars* are ±1 SD. When identical droplets are moved across the native device surface or an immobilized spot of BSA, the non-specific adsorption is very low in comparison to specific binding to IgG (*inset*)

and after the assay (not shown) indicate that the bulk droplet retained much more FITC-anti-human IgG than was bound by the surface—thus, the size of the spot and density of antigen are the limiting factors under these conditions. Measurements were also collected from unmodified spots on the device surface and from spots containing BSA to determine the extent of non-specific binding to the device surface (Fig. 3 inset).

The results in Fig. 3 are instructive for DMF protocol development. Since flow within this DMF system is laminar (the Reynolds Number is ~210), it is likely that the spot captures all of the antibody from the layer of fluid in direct contact with the surface. We speculate that the binding capacity of spots in such devices could be increased by mixing the droplet on a separate electrode array or via pulses of applied potential in between passes. Furthermore, it is evident that subsequent passes of the droplet do not decrease the amount of bound antibody despite the presence of Pluronic in the droplet. For subsequent experiments, ten droplet passes were used for each binding step of the assay. (Even though two passes appear to be adequate for antigen/antibody binding to take place, more passes were used such that variations in fluid movement or exposure time can be eliminated as a source of variance in the data.) Two passes were used for each rinse step to remove non-specifically bound protein in between steps of the assay.

Optimized immunoassay The optimized immunoassay protocol was used to analyze antigen standards of varying concentrations. A conventional 96-well plate assay was also performed with the same concentrations as a comparison. As shown in Fig. 4, the calibration curves for the two methods are quite similar. While the relative variances in the conventional scale method are slightly smaller than those in the DMF method, the calibration curve generated on-chip is more linear, and each data point generated by DMF has a RSD less than 25%. As we have observed previously,[30] the difference in the variances between the two methods most likely arises from manual pipetting of 1 μL droplets of solutions onto the DMF platform (in both the printing step and the sample/reagent dispensing steps) and not from the DMF fluid handling itself. As this work was primarily intended as proof-of-principle of the heterogeneous assay itself, automated fluid handling was not used; however, we expected that, with the addition of automated fluid dispensing in both the printing steps and the dispensing of solutions on-chip, these errors will be reduced.

In these experiments, the use of multiplexed DMF devices (Fig. 1b) comprising six to ten platforms wired in series and operated simultaneously facilitated the standardization of droplet actuation (and thus, the fluidic conditions of the assay) across replicates of the same measurement. Since the amount of antigen and detection antibody bound by each spot is dependent on the movement of the droplet, simultaneous handling of replicate samples eliminates some of the human error inherent in manual droplet control.

The linearity of the data in Fig. 4 indicates that this assay can serve as a quantitative tool, particularly if the fluid handling can eventually be fully automated. Both the DMF-driven and well-plate method have a dynamic range of approximately one order of magnitude—this is typical for

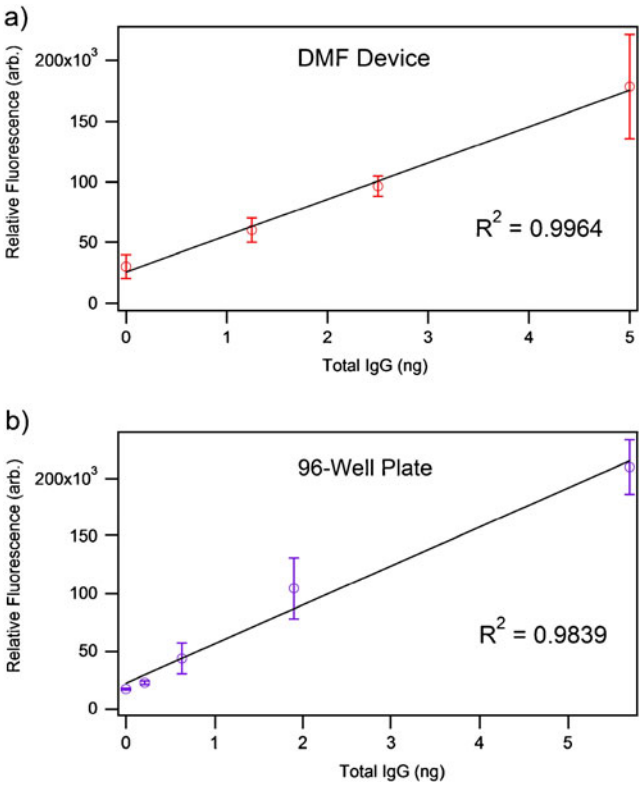

Fig. 4 Standard curves for DMF (**a**) and 96-well plate (**b**) immunoassays. Both methods have a linear dynamic range of approximately one order of magnitude, and similar variances in data. The DMF method shows a stronger linear correlation, but has slightly larger variances (%RSD<25%). Each *point* represents three to five replicate trials and the *error bars* are ±1 SD

surface-based immunoassays, and is primarily limited by the sensitivity of the detector being used. The saturation point of the assay was not found before the fluorescence detector was saturated, so it is possible that a broader dynamic range could be achieved by using a less sensitive detector. However, this dynamic range is well suited for examining the variations in IgG concentrations (less than one order of magnitude [31]) that might indicate illness or variation between individuals.

There are two major advantages of the digital microfluidic assay: a dramatic reduction in analysis time, and reduced consumption of reagent volumes. As context for the former advantage (analysis time), a conventional immunoassay on a multiwell plate requires up to 2 days from start to finish (up to 12 h for capture antibody immobilization, 2–12 h for BSA blocking steps, and 1–2 h for each of the antigen and detection antibody binding steps). In contrast, the DMF assay requires only 2.5 h from printing of the spots to completion. The major advantage in analysis time is a result of the dynamic nature of the DMF assay—delivery of antigen to the capture antibody is not achieved solely by diffusion. Incubation times are reduced because of diffusion distances that are two orders of magnitude smaller, coupled with the motion of the droplet. In addition to the efficiency of active transport to the device surface, the time course of the DMF method benefits from the elimination of the BSA blocking step on the DMF device—a step that cannot be eliminated in a well plate. As higher throughput DMF devices are developed, this analysis time will decrease further as a greater number of replicates and samples can be processed simultaneously. For the latter advantage (reagent consumption), sample and reagent concentrations were chosen such that the DMF method would consume the same absolute mass of antibody and antigen as the published well-plate protocol for purposes of comparison. However, it should be noted that the detector was operated at a much lower gain using the DMF method. Preliminary experiments have shown that the DMF assay can be performed at concentrations at least an order of magnitude lower than those shown here without sacrificing reproducibility or dynamic range (data not shown)—the absolute limits of detection and quantitation have not yet been determined. In addition, total waste volumes have been reduced by three orders of magnitude, from approximately 3.5 mL/well to 4 μL/DMF spot.

Analysis of complex samples To determine the feasibility of applying the DMF method to real samples as well as the likelihood of cross-reactivity with interfering species, samples of bovine serum were doped with analyte (human IgG) and analyzed. The very high protein concentration of proteins in serum makes it difficult to move on a DMF platform,[29] so in this work, the serum was diluted by two orders of magnitude, resulting in a bovine IgG concentration of approximately 100 μg/mL.[32] Thus, the concentration of bovine IgG in these tests was an order of magnitude higher than the highest concentration of analyte (human IgG) doped into the system. As shown in Fig. 5, the

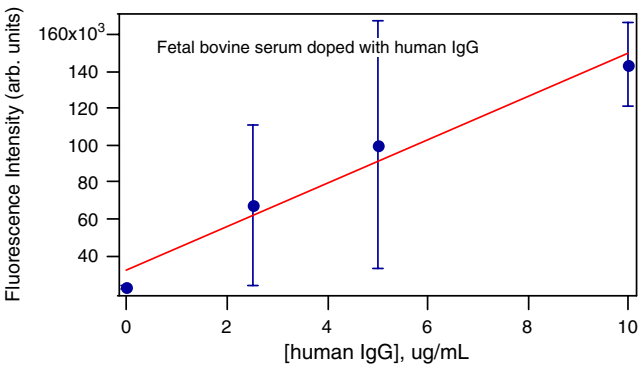

Fig. 5 Quantitation of human IgG in bovine serum samples (diluted 100×). Large variances are likely a result of reduced reproducibility in droplet movement at high protein concentrations. Binding of human IgG of various concentrations is evident, and a linear standard curve was obtained with a dynamic range of one order of magnitude, despite the presence of bovine IgG at a tenfold higher concentration in the sample

calibration curve has large variance in the data, but it is clear that the analyte can be detected in a complex matrix. The assay shows no signs of significant cross-reactivity with bovine IgG, as its much higher concentration in the sample would saturate the assay spot in every sample, drowning out the signal from the added human IgG. The large errors are attributed to the high protein concentrations in the sample, which interfere with droplet movement and diffusion of the analyte to the surface for binding. Regardless, these results are encouraging, as they indicate that this assay is capable of detecting a low-concentration analyte in a complex sample matrix. This result is promising for the future application of DMF immunoassays to serum samples—we speculate that a more reproducible method for printing capture antibody spots such as microcontact printing or an inkjet-style protein spotter will reduce the variance in spot size, and propose that high-throughput devices should standardize the fluid handling, specifically dispensing.

Conclusion

We have developed a digital microfluidic platform that can be used to carry out a heterogeneous immunoassay directly on a device surface, without need for beads, BSA blocking, or a carrier oil interface for droplet movement. Capture antibodies were successfully immobilized on the DMF device, and conditions were optimized for fluid movement and assay execution. The need for BSA blocking of the assay surface was eliminated by the use of Pluronic F-127 additives to prevent non-specific binding of analyte to the surface. As a result, IgG molecules were detected and quantified in sample volumes 100-fold smaller than those used in well-plate assays, while reducing analysis times by an order of magnitude. The data were reproducible, and no loss in dynamic range was observed during the translation to microscale. IgG was also successfully detected in complex serum samples containing interfering proteins. We conclude that this method has great potential as a high-throughput, low-waste, and inexpensive technique for the quantitative analysis of macromolecules.

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