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A Miniaturized, Parallel, Serially Diluted Immunoassay for Analyzing Multiple Antigens

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This report describes a miniaturized, microfluidic version of a serial-dilution fluorescent immunoassay.[1] This assay is capable of analyzing multiple antibodies quantitatively and in parallel in small volumes (<1 μL) of liquids in one experiment. It uses a network of microfluidic channels to achieve serial dilution; we call this system of microchannels a microdilutor network (μDN, Figure 1). The branching structures of the μDN serially dilute one stream with a second (so long as proper mixing occurs at each stage). Flow in microchannels is normally laminar;[2] to ensure complete mixing, we incorporate chaotic advective mixers (CAMs) into the μDN.[3] In this assay, we use this device to dilute a sample serially with buffer.

We illustrate this assay by determining the concentrations of antibodies (IgGs in this case) in HIV+ human serum (anti-gp41 and anti-gp120). In the assay, serially diluted solutions of serum flow in channels across orthogonal, parallel strips of HIV ENV proteins (antigens, gp41, and pg120) adsorbed on a polycarbonate membrane. The soluble antibodies bind to these adsorbed antigens and are themselves immobilized; the quantity of adsorbed antibody can be measured using a second, fluorescent antibody.

We believe that this method provides a new and general approach to one of the most common bioanalytical procedures.[1] The μDN replaces the set of microwells used in manual serial dilutions; the format of this assay is the same as that of the traditional ELISA-type assays for HIV; this format includes the sequential adsorption and immobilization of antigen, antibody, and secondary antibody.[4] This method is, we believe, generalizable to the analysis of 10−100 antibodies (as long as there is no cross-reactivity between these antibodies), although we have analyzed only two.

The microfluidic device has two components (Figure 1): the first component is the μDN that dilutes the analyte (serum containing HIV antibodies) serially using CAMs (the top portion of Figure 1) to achieve mixing; the second component is a membrane that presents stripes of immobilized proteins (in this case, gp41 and gp120; bottom portion of Figure 1 and Supporting Information Figure S1). The dilutor mixes and dilutes the serum with a buffer containing 5% bovine serum albumin (BSA), used to block nonspecific adsorption of antibodies on the surfaces of the device); this procedure generates a series of solutions containing exponentially decreasing concentrations of antibodies. The design in Figure 1 achieves 1:1 mixing of serum-containing solution and buffer in each stage of dilution. Each stage thus decreases the concentration of antibodies by one-half. (Supporting Information Figure S2 quantifies the efficiency of mixing in the device.) Using 1:1 dilutions and 10 sequential stages of mixing and dilution, we have achieved a dynamic range of 2^{10}∼10^3. A 1:3 dilution would, in principle, achieve a dynamic range of 10^6 with the same number of stages of mixing and splitting; we have not yet demonstrated this range experimentally. We demonstrated the function of the dilutor using BSA conjugated to fluorescein (BSA-FITC) and

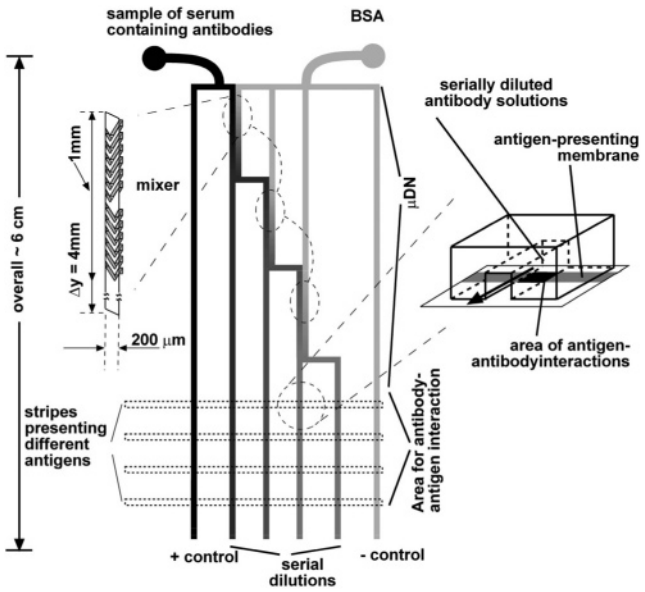

Figure 1. Schematic representation of the analytical device. The μDN (top of figure) generates a serial dilution of analytes that cross over immobilized antigens. Each CAM includes four cycles of herringbone patterns and is 4 mm long; one cycle is shown in an expanded view. The bottom part of the figure suggests how the serially diluted antibody interacts with spatially segregated antigens, immobilized on a polycarbonate membrane.

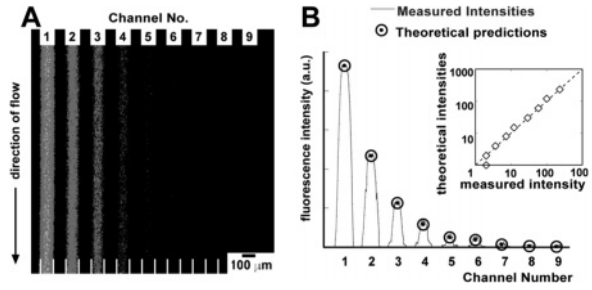

Figure 2. The on-chip dilutor serially dilutes BSA-FITC. (A) A serial dilution network that dilutes a total of nine channels, where a 1:1 mixing is achieved serially. (B) The graph shows the measured fluorescent intensities in all nine channels (indicated on the *x* axis). The insert shows the correlation between measured and calculated intensities across a dynamic range of almost 10^3.

phosphate buffer saline (PBS) in solution (Figure 2); observed and expected values agree well.

The polycarbonate membrane (the second component of the system) presented the antigens patterned in microstripes (bottom portion of Figure 1, Supporting Information Figure S1A and C; assays based on similar principles have been demonstrated previously[5,6]). We used an array of microchannels to deliver gp120 and gp41 to a polycarbonate membrane.[5] The membrane contained pores of diameter ∼200 nm; these pores presented large surface areas

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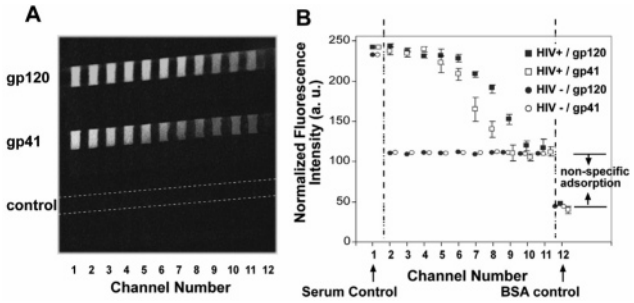

Figure 3. Results of the assay. (A) shows a micrograph of the membrane used in this assay. (B) Plots of the intensities obtained from (A). Also shown are the fluorescent intensities of the control experiments using HIV− serum, using the same antigens as the HIV+ experiments. The error bars represent the range of the fluorescent intensities collected from at least three independent samples.

that both adsorbed amounts of proteins sufficient to give good sensitivity and also maintained the proteins in hydrated and active forms when the membrane was momentarily dried during assembly of the system. The antigen-immobilized membrane was sandwiched between the two pieces of PDMS that formed the microchannels. (See the Supporting Information for the details of the fabrication and assembly of the device.) When serially diluted serum containing anti-gp120 and anti-gp41 flowed orthogonally across the stripes of adsorbed antigens, the antibodies in the serum bound to these antigens.

The amount of antibody that bound to the immobilized antigens depended on its concentration in solution. We quantified bound antibodies using a second, fluorescent antibody (Figure 3, see the Supporting Information for details). The serial dilution experiments showed that the serum was positive for both anti-gp120 and anti-gp41: fluorescent signals decreased with the concentration of antibody between channels 5 and 9. (In all experiments, channel 1 was a positive control: the sample of serum was allowed to flow through a distinct channel without dilution.) In channels 1−4, the fluorescent signals were saturated for both anti-gp120 and anti-gp41. In channels 10 and 11, the intensities of signals no longer decreased; we infer that the level of signal detected in these channels represented nonspecific adsorption. Channel 12 was the negative control and contained only the dilution buffer. The difference in signal intensity between channels 11 and 12 reflected nonspecific binding on the membrane.

This assay was kinetic, rather than equilibrium. It comprised two parts. In the first, the antigens adsorbed irreversibly and uniformly on the polycarbonate membrane (Supporting Information Figure S3C). Exposure of adsorbed gp120 and gp41 to flowing buffer established that no desorption occurred between 15 and 300 min (between 2.7 and 54 μL of solutions, or between 30 and 240 min at 3 nL/s) (Supporting Information Figure S3). In the second part of the assay, binding of anti-gp120 and anti-gp41 onto the membrane-bound antigens was also irreversible, but the *amount* bound depended on both the concentration and the time of exposure to the solution containing serum. Upon exposure to a sufficient quantity of this solution (>12 h of flow, at 3nL/s, a total of 130 μL of serum), even the channels with the lowest concentrations of antibodies saturated the surface (Supporting Information Figure S4A). At low concentrations of antibodies, however, this binding was slow. Once the antibodies had adsorbed to the surface, they did not dissociate: washing a membrane having adsorbed antibodies with a flow of PBS for >12 h (a total of 130 μL) did not decrease the intensities of the signals (Supporting Information Figure S4C). Thus, within appropriate ranges of concentration for solutions of antibodies and conditions of flow (between 5.4 and 43 μL of solutions, or between 30 and 240 min at 3 nL/s, for the series of concentrations present in the sample), the quantities of anti-gp120 and anti-gp41 that bound to the surface only depended on the concentrations of antibodies in solution.

Once the amount of antibody on the surface is known, it is possible to find the concentration of antibody in the solution. To estimate the absolute concentrations of these antibodies in solution, we used a standard curve that related concentrations of human IgGs (a mixture of IgGs isolated from human plasma, hIgG) in solution to concentrations of hIgG on the polycarbonate membrane. (The generation of the standard curve and the calculation of the concentrations of the hIgG are described in the Supporting Information.) We estimated the amount of anti-gp120 and anti-gp41 in the serum to be 2.0 ± 0.7 and 1.0 ± 0.5 mg/mL, respectively.[7]

This assay demonstrates a general format for miniaturized serial dilution assays. We believe that parallel, quantitative assays of multiple analytes, using small volumes of sample, will find uses in clinical, pharmaceutical, and environmental sciences. We have used PDMS in these initial experiments because it is particularly easy material with which to prototype.[8] The same design, made of other polymers, or of glass, can also be used. When combined with miniaturized detection systems, this design allows simultaneous, quantitative analysis of multiple analytes to be carried out on a single chip.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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