UC Berkeley

UC Berkeley Previously Published Works

Title

Microfluidic barcode assay for antibody-based confirmatory diagnostics.

Permalink

<https://escholarship.org/uc/item/0v041077>

Journal

Lab on a chip, 13(19)

ISSN

1473-0197

Authors

Araz, M Kursad
Apori, Akwasi A
Salisbury, Cleo M
et al.

Publication Date

2013-10-01

DOI

10.1039/c3lc50229e

Peer reviewed

PAPER

Microfluidic barcode assay for antibody-based confirmatory diagnostics†

Cite this: *Lab Chip*, 2013, 13, 3910M. Kursad Araz,^a Akwasi A. Apori,^a Cleo M. Salisbury^b and Amy E. Herr^{*ac}

Confirmatory diagnostics offer high clinical sensitivity and specificity typically by assaying multiple disease biomarkers. Employed in clinical laboratory settings, such assays confirm a positive screening diagnostic result. These important multiplexed confirmatory assays require hours to complete. To address this performance gap, we introduce a simple 'single inlet, single outlet' microchannel architecture with multiplexed analyte detection capability. A streptavidin-functionalized, channel-filling polyacrylamide gel in a straight glass microchannel operates as a 3D scaffold for a purely electrophoretic yet heterogeneous immunoassay. Biotin and biotinylated capture reagents are patterned in discrete regions along the axis of the microchannel resulting in a barcode-like pattern of reagents and spacers. To characterize barcode fabrication, an empirical study of patterning behaviour was conducted across a range of electromigration and binding reaction timescales. We apply the heterogeneous barcode immunoassay to detection of human antibodies against hepatitis C virus and human immunodeficiency virus antigens. Serum was electrophoresed through the barcode patterned gel, allowing capture of antibody targets. We assess assay performance across a range of Damkohler numbers. Compared to clinical immunoblots that require 4–10 h long sample incubation steps with concomitant 8–20 h total assay durations; directed electromigration and reaction in the microfluidic barcode assay leads to a 10 min sample incubation step and a 30 min total assay duration. Further, the barcode assay reports clinically relevant sensitivity (25 ng ml^{-1} in 2% human sera) comparable to standard HCV confirmatory diagnostics. Given the low voltage, low power and automated operation, we see the streamlined microfluidic barcode assay as a step towards rapid confirmatory diagnostics for a low-resource clinical laboratory setting.

Received 19th February 2013,
Accepted 17th July 2013

DOI: 10.1039/c3lc50229e

www.rsc.org/loc

Introduction

Confirmatory diagnostic assays are critical to eliminating false positive screening results. In hepatitis C virus (HCV) or human immunodeficiency virus (HIV) diagnosis, the US Centres for Disease Control and Prevention (CDC) recommend a screening enzyme immunoassay (EIA or ELISA) with subsequent confirmation of a positive result using a Western blot or immunoblot.^{1–3} To provide higher clinical sensitivity and specificity than rapid screening assays, confirmatory blots assay multiple biomarkers in a single sample. Yet, even in developed nations, lack of funding is cited by public health centres as limiting access to HCV diagnostics.^{3–5} When considering less developed nations, the access to confirmatory diagnostics is even more limited.^{6,7} Accessibility limitations of confirmatory blotting assays stem, in part, from copious

resource consumption. In conventional formats, the assays are slow, labour intensive, and costly; requiring laboratory infrastructure and trained staff.^{8–10}

The *de facto* standard for HCV confirmatory diagnosis is the multistage Recombinant Immunoblot Assay (RIBA). For the heterogeneous RIBA immunoassay, HCV antigens are pre-immobilized in bands along a membrane strip. To make a HCV infection diagnosis, serum is incubated with the RIBA strip; RIBA-based detection of serum antibodies against the HCV antigens informs final diagnosis. The RIBA assay typically runs 6–8 h.^{8,10} The diffusion-based transport of serum antibodies to immobilized HCV antigens on the RIBA membrane is a key rate limiting step.

Consequently, assay developers have sought to introduce microfluidic systems with directed, active transport to expedite heterogeneous immunoassays.^{11,12} Researchers have used myriad approaches including channel surface functionalization,¹³ packed bead beds,¹⁴ magnetic beads,¹⁵ and functionalized polymers¹⁶ to name a few. Bimolecular capture efficiencies are both controlled and enhanced in these systems,^{17–19} as compared to diffusion-based passive antibody detection (*i.e.*, RIBA). Yet, microfluidic heterogeneous immunoassays specifically developed for immunoglobulin detection

^aDepartment of Bioengineering, University of California, Berkeley, Berkeley, CA, USA

^bNovartis Diagnostics, Emeryville, CA, USA

^cUniversity of California, Berkeley—University of California, San Francisco Graduate Program in Bioengineering, University of California, Berkeley, CA, USA.

E-mail: aeh@berkeley.edu

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3lc50229e

are limited.^{20–24} A significant challenge stems from the high concentration levels of total immunoglobulin in serum (6–18 mg ml^{−1}).^{25–27} Underpinning the high clinical sensitivity and specificity of HCV blotting confirmatory diagnostics is the capacity to detect multiple immunoglobulin molecules in one biospecimen (*i.e.*, multiplexing).

Microfluidic multiplexing has been accomplished in heterogeneous immunoassays *via* surface patterning^{21,28,29} and custom functionalized beads³⁰ or in homogeneous immunoassays utilizing electrophoresis in microfluidic channel networks.³¹ Relevant to the present study, functionalization of channel-filling polymer gels brings together advantages of homogeneous and heterogeneous assays.³² The reactive, functionalized gels provide fixed reagent capture sites distributed throughout the channel bulk, thus yielding a pseudo-homogeneous assay.^{33–36} Enhancing the capture site and reagent interactions dramatically, these pseudo-homogeneous approaches reduce interaction distance and the time required for diffusion-based capture.

Nevertheless, the majority of microfluidic multiplexing platforms rely on complex control channel networks for fabrication and operation.^{29,35,37} Multiple channel networks increase the device footprint and operation complexity. These approaches use multiple polymerization steps for custom creation of regions leading to discontinuities in the gel interfaces. In serological assays, gel discontinuities lead to nonspecific capture of highly abundant immunoglobulin and inherent nonspecific signal.

We introduce an HCV confirmatory blotting assay appropriate for rapid completion in clinical laboratories – importantly, confirmatory diagnoses are conducted under the supervision of a healthcare professional, making point-of-care use typically inappropriate. Assay speed remains important in laboratory settings as rapid diagnoses can immediately direct treatment of infectious conditions. Further, resource allocation in clinical chemistry laboratories benefits from fast assays. We develop a simple single straight microchannel and a ‘single inlet, single outlet’ electrophoretic fabrication approach to allow barcode patterning of multiple capture antigens along the length of one channel. We pattern distinct capture reagents (antigens) and non-reactive spacer regions in the 2.5 mm long channel. The multiplexed microfluidic barcode assay is demonstrated for detection of immunoglobulin specific for HCV-c100p, HCV-NS3 and HIV-I p24 antigens—a multi-biomarker panel for diseases with high co-infection rates. The single microchannel barcode assay completes in 30 min with an analytical sensitivity and specificity comparable to the 6+ hour RIBA 3.0 assay. Sources of fabrication and assay variability are discussed. Importantly, assay operation is purely electronic at the one inlet and one outlet, with electrophoresis conducted at low applied voltages, thus eliminating the dependence on high voltage power supplies, pumps, syringes, or valves—making the device appropriate for rapid, facile operation.

Materials and methods

Reagents

HCV positive pooled human plasma derived HCV core, NS3 and c100p antibodies (AF-568 conjugated and unconjugated), and recombinant HCV core, NS3 and c100p antigens and their biotin and AF488 conjugated formats were provided by Novartis Diagnostics (Emeryville, CA). HIV p24 antigen and HIV p24 mouse antibodies were purchased from Prospec-Tany Technogene (Rehovot, Israel). Biotin conjugated protein-L was purchased from Thermo Fisher Scientific (Rockford, IL). Biotin and Biotin-FITC were purchased from AnaSpec (Fremont, CA). Streptavidin-acrylamide, and AF-568 conjugated anti-human and anti-mouse secondary goat antibodies were purchased from Invitrogen (Carlsbad, CA). HCV-HIV negative human sera were purchased from SeraCare Life Sciences (Oceanside, CA). All antibody, antigen and serum samples were diluted in 1 × Tris-glycine (TG) buffer purchased in 10 × strength from Bio-Rad (Hercules, CA). VA-086 photoinitiator (2,2-azobis [2-methyl-*N*-(2-hydroxyethyl) propionamide]) was purchased from Wako Chemicals (Richmond, VA). 30 : 1 Acrylamide-bisacrylamide monomer solution, glacial acetic acid, methanol and 3-(trimethoxysilyl)-propyl methacrylate (Silane 174) were purchased from Sigma-Aldrich (St. Louis, MO).

Imaging, data acquisition and chip operation

Images were captured *via* a Peltier-cooled CCD camera (Cool Snap HQ2, Roper Scientific, Trenton, NJ) attached to an Olympus IX-50 epi-fluorescence microscope (Center Valley, PA) equipped with 10 × objective (UPlanFL, N.A. = 0.3, Olympus, Center Valley, PA), and GFP (41017 FL-CUBE ENDOW) and DSRED (XF111-2-CSM; OMEGA FL) filters (Omega Optical, Battleboro, VT). A 100 W mercury arc lamp was used as the excitation source. Semi-automated image capture was controlled through Metamorph software by Molecular Devices LLC (Sunnyvale, CA) installed on a PC (Dell Inc. Round Rock, Texas). Exposure times with 4 × 4 pixel binning varied between 5 ms–400 ms depending on the experiment. Post-processing and analysis of the images were performed with ImageJ (NIH, Bethesda, CA) and Matlab (Mathworks, Natick, MA). A custom built, computer controlled, 8 channel current or voltage programmable high voltage power supply (HVPS) was used.³⁸ Quantization of the assay results was achieved by calculating the area under the curve (AUC) values of the fluorescence intensity profiles obtained along the channel. Both the intensity profile extractions and the total signal integration for the selected region of interest (ROI) were performed in ImageJ. To eliminate photobleaching based intensity variation during the final antibody signal readout, extra care was taken to keep number of exposures and exposure times identical for each channel within an experiment set.

Chip and gel fabrication

Chips were designed in-house and fabricated in soda lime glass substrates by Caliper Life Sciences (Hopkinton, MA) utilizing standard wet silica etching methods and thermal bonding. Seven straight channels were housed on each chip (Fig. 1A). Channels were 2.5 mm long, 35 μm deep and 120 μm

Fig. 1 Microfluidic barcode assay for multiplexed protein detection. (A) A 17.5 mm $\times$ 17.5 mm glass chip with six simple straight (2.5 mm long) microchannels, each housing a streptavidin-PA gel. (B) Fluorescence micrograph of four c100p-AF488 antigen bands and interlaced biotin spacers. (C) Electrophoretic patterning process consists of: (steps 1–3) Biotin blocking to create an entry area spacer *via* electrophoretic load, incubation, and reversed polarity field to remove unbound biotin, (steps 4–6) Patterning of biotinylated antigen after spacer region *via* electrophoretic introduction, 1 min incubation and reversed polarity electrophoretic removal of unbound biotinylated antigen. Steps 1–6 are repeated for each subsequent reagent-spacer pair pattern.

wide. Two millimetre diameter, 1 mm deep reservoirs for sample and electrical connectivity were located at each channel terminus.

Before PA gel fabrication, channel walls were functionalized for enhanced gel to wall attachment as previously reported.^{16,39} Slight modifications include: before the func-

nalization step, channels were cleaned with 1 M NaOH for 10 min, followed by 3 DI-water wash steps. To next generate the silane monolayer coating on the channel walls, channels were incubated with a premixed, degassed and agitated solution of 2 : 3 : 2 silane-water-acetic acid for 30 min. Incubation was performed at room temperature with the chip sitting in a covered petri dish housing a wetted tissue to increase humidity and limit evaporation. After monolayer formation, silane solution was washed out of the channels with methanol for 5 min followed by two rounds of methanol and then water washes. After the final wash step, channels were aspirated and then purged with N_2 gas. Gel precursor solution was prepared in 1 $\times$ TG buffer with 3%T acrylamide/bisacrylamide (29 : 1), 4 μM streptavidin acrylamide (SA) and 2 mg ml^{-1} VA-086 photoinitiator. Degassed and agitated solution (10 min sonication with vacuum applied to the tube) was wicked into the microchannels in 1–2 s. Polymerization of streptavidin-PA gel was accomplished *via* flood UV exposure with a UV lamp (UVP B100-AP, Upland, CA) enclosed in a home-built air-cooled box for 10 min at 10 mW cm^{-2} . The chip was placed in a humidified petri dish. Following polymerization, chips were visually inspected for gel defects. If defects in the gel were detected, the chip was discarded. After polymerization, excess gel in the wells was removed with a pipette tip attached to vacuum. Chips were stored at 4 $^{\circ}\text{C}$ in a humidified petri dish, as described. Following the completion of experiments, chips were disinfected with 5% bleach solution. Gel was removed using a perchloric acid-hydrogen peroxide solution used with appropriate safety controls.¹⁶

Antigen immobilization and assay operation

After gel preparation, the streptavidin-PA gel-filled microchannels were electrophoretically patterned with barcode-like patterns of biotinylated antigens (Fig. 1B). An overview of the patterning process is in Fig. 1C and Table S1, ESI.[†] Similar to the reagent-spacer patterning, assay operation was performed electrophoretically as described in Fig. 2 and Table S2, ESI.[†] Human serum diluted in TG buffer was electrophoretically delivered to the immobilized antigen zones. As target antibodies electromigrated through the reagent patterned gel, target antibodies were captured in the corresponding antigen zone. After flushing antibody out of the channel, fluorescently labelled secondary anti-human antibodies were introduced for optical detection of bound primary antibodies.

Results and discussion

Competition between reaction and electromigration underpins fabrication of barcode format

We sought to determine the feasibility of a purely electrophoretic heterogeneous immunoassay in a one inlet and one outlet microchannel: a microfluidic “barcode” assay (Fig. 1A). In this heterogeneous immunoassay the immobilized phase is a capture protein used to probe for antibody in a biospecimen. The fabrication and assay are designed to be controlled using only an applied electric potential (*i.e.*, no pumps, valves). The patterning principle invokes two phenomena expected when

Fig. 2 Schematic of antibody sandwich assay: Serum sample electrophoretically delivered to the channel sieves through immobilized antigen zones where corresponding target antibodies are captured. In a following step, detection is enabled with fluorescently labeled antihuman secondary antibodies loaded electrophoretically. Bottom images show the antibody signal from a 2% human serum sample spiked with $1 \mu\text{g ml}^{-1}$ HCV-c100p antibody. Fluorescence detection is enabled by AF-568 labeled antihuman goat antibodies.

biotinylated proteins electromigrate through a channel-filling streptavidin-PA gel. Namely, that the biotinylated capture protein will either freely electromigrate along the channel axis (e.g., in cases where the streptavidin-PA gel is already saturated with biotin, no patterning of capture protein) or the biotinylated capture protein will bind to streptavidin resulting in protein immobilization (patterning) (Fig. 1B). The multiplexed barcode patterning strategy reported here takes advantage of both processes to create patterned antigen regions interleaved with non-reactive ‘spacer’ regions. Spacers are created by biotin saturation of streptavidin to ‘block’ subsequent immobilization of biotinylated capture proteins.

Quantitatively, we can describe the different behaviours used to fabricate the barcode pattern by considering the competition between electromigration and biotin-streptavidin binding that yields either protein migration along the axis (no binding) or immobilization of biotinylated protein in the gel (binding). To describe the behavioural regimes, we formulate a Damkohler number^{40,41} (Da) comparing the reaction time scale ($t_r = k_{\text{on}}^{-1} \cdot b_m^{-1}$) to the electromigration time scale ($t_t = L/u_i$), where $u_i = \mu E$ through the relationship $Da = t_t/t_r = Lk_{\text{on}}b_m/\mu E$. Here, k_{on} is the association constant, b_m is the concentration of the available capture sites, L is the characteristic length of the capture region, u_i is the speed of the electromigration, μ is the electrophoretic mobility and E is the electric field.

When $Da < 1$, reaction is slow compared to electromigration and binding is limited by the reaction speed. In this regime, electromigration of proteins is so fast (compared to the binding kinetics) through the streptavidin-PA gel that biotinylated capture proteins do not spend enough time in the neighbourhood of a streptavidin binding site to become immobilized. Alternately, there may be so few streptavidin binding sites available ($b_m \rightarrow 0$) that biotinylated proteins move along the axis of the channel without becoming immobilized.

At the other extreme, where $Da > 10$, the binding reaction is mass transport limited such that biotin-streptavidin reaction kinetics are faster than the electromigration timescale. Thus, we anticipate that the immobilization efficiency will be high in mass transport limited operation. For barcode patterning, operation in the mass transport limited regime results in successful immobilization of biotinylated protein to the streptavidin-PA gel. The range of $1 < Da < 10$ is expected to be a transition region between the two extreme regimes.

Barcode patterning in a single inlet/single outlet channel relies on a sequence of patterning/blocking steps to create the spacer regions and the capture protein regions (Fig. 1B, C). Firstly, to create the spacer regions, we saturate the streptavidin-PA gel sites with high concentrations of free biotin ($b_m \rightarrow 0$). These regions thus allow subsequent electromigration of biotinylated capture proteins through the non-reactive gel and down the channel axis. Secondly, to pattern regions of capture reagent, we operate under conditions in which streptavidin is plentiful in the PA gel. Biotinylated proteins are electrophoresed into the channel and immobilized at open streptavidin sites in the gel matrix. The biotinylated proteins populate streptavidin sites in an ordered fashion, starting at the reservoir of origin. Electromigration of biotinylated protein along the channel – without immobilization – is possible when the local streptavidin becomes saturated or when transport outspeeds reaction. By alternating spacer and capture protein patterning, a single inlet/single outlet microchannel supports fabrication of a multiplexed barcode assay comprised of spacer regions between different capture antigens.

Patterning of alternating capture protein and spacer regions

Barcode patterning in a single inlet/single outlet channel relies on a sequence of patterning/blocking steps to create the spacer regions and the capture protein regions (Fig. 1B, C). Firstly, to create the spacer regions, we saturate the streptavidin-PA gel sites with high concentrations of free biotin ($b_m \rightarrow 0$). These regions thus allow subsequent electromigration of biotinylated capture proteins through the non-reactive gel and down the channel axis. Secondly, to pattern regions of capture reagent, we operate under conditions in which streptavidin is plentiful in the PA gel. Biotinylated proteins are electrophoresed into the channel and immobilized at open streptavidin sites in the gel matrix. The biotinylated proteins populate streptavidin sites in an ordered fashion, starting at the reservoir of origin. Electromigration of biotinylated protein along the channel – without immobilization – is possible when the local streptavidin becomes saturated or when transport outspeeds reaction. By alternating spacer and capture protein patterning, a single inlet/single outlet microchannel supports fabrication of a multiplexed barcode assay comprised of spacer regions between different capture antigens.

To assess the patterning strategy, we fabricated a barcode assay comprised of spacer regions and biotinylated c100p as the capture protein. Fig. 3A reports a patterning time course. Prior to introduction of biotinylated c100p capture protein, a small spacer region was fabricated at the channel inlet, identified as region (i) in Fig. 3A. After patterning of the spacer region, the polarity of the electric potential was reversed and unbound biotin was electrophoresed out of the channel. After reservoir flushing *via* aspiration, patterning of capture protein commenced with biotinylated c100p antigen electromigrating into the channel. As observed in Fig. 3A, biotinylated c100p migrated through the spacer region (i) with negligible immobilization of c100p. As c100p entered the unblocked

Fig. 3 Characterization and optimization of the electrophoretic barcode patterning process. (A) Time lapse fluorescence micrographs (10 s/frame) of patterning of biotinylated c100p antigen (AF-488 labelled). Region (i) was previously blocked with biotin (spacer). Region (ii) is the region being patterned with the biotinylated c100p. At $t = 140$ s, the electric field is removed and incubation commences. After a reverse polarity electrophoretic wash, Region ii has been patterned with the c100p and the open streptavidin-PA gel sites in Region iii are available for subsequent patterning with biotin spacer or biotinylated antigen. (B) Patterning process as viewed through breakthrough isotherms of axial fluorescence intensity (biotinylated c100p, AF-488 labelled). First 11 curves at 5 s increments from $t = 0$. (C) Fluorescence micrographs show antigen immobilization at various Da numbers. Fluorescence intensity plot of moving boundary and first derivative are shown below images for $Da = 183$. (D) Width (4σ) of moving boundary for patterning at various Da numbers. Fluorescence intensity vs spatial location data in (B, C, D) were obtained in ImageJ through the extraction of intensity values along the central axis of the channel.

streptavidin-PA gel region (iii), the biotinylated c100p capture protein became immobilized and started forming region (ii). Fabrication conditions were estimated to be $Da \sim 60$. To allow real time monitoring of both the length and location of the patterned protein bands, trace amounts of dual AF-488 and biotin labelled c100p protein were mixed with biotin labelled c100p protein at a 1 : 100 ratio.

As shown in Fig. 3B, as the capture protein concentration front migrated along the channel axis; a self-sharpening behaviour at the interface between regions (ii) and (iii) with enrichment in the protein concentration in region (ii) was observed. Reminiscent of breakthrough isotherm curves observed in adsorption processes in packed columns, self-sharpening behaviour is attributed to a favourable adsorption dynamics with fast capture kinetics.⁴¹ Saturating the available sites, immobilized protein concentration was expected to be enriched by a factor of n , where n is the ratio of the concentration of the available capture sites to the concentration of the biotinylated proteins to be immobilized in the gel. The 3.4 ± 0.2 fold increase in the fluorescence signal in region (ii) agrees with the enrichment factor for the given 5 μ M biotinylated c100p protein and 4 μ M streptavidin (*i.e.*, providing 16 μ M biotin capture site, $16/5 = 3.2$). The electrophoretic velocity u_i is $u_i = \mu_i E$ where μ_i is the electrophoretic mobility of the protein sample in a given gel medium and E is the applied electric field. From conservation of mass, the velocity of the moving boundary in region (ii) v_i is expected to decrease with the order of the enhancement factor n , resulting $v_i = u_i/n = \mu_i E/n$. From Fig. 3B the reduction in velocity is by a factor of 3.5 ± 0.5 which is in agreement with the increase observed in the fluorescence signal (3.4 ± 0.2).

With continued electromigration of biotinylated c100p capture protein from the reservoir, the boundary of the immobilized capture protein region penetrated further along the microchannel axis, yielding a longer axial extent of immobilized capture protein, Δx_i (Fig. 3A). This distance Δx_i depends on the velocity of the expanding boundary v_i , and the duration of the injection, Δt_i ($\Delta x_i = v_i \Delta t_i = \mu_i E \Delta t_i / n$) and can be optimized for each capture protein by controlling the duration of the loading process or applied electric field. For instance, as shown in Fig. 3A at 10 V drive (40 V cm^{-1}), patterning of an 800 μ m long region with c100p antigen loaded at 5 μ M was achieved within 140 s. For comparison, the same patterned length was achieved in 3 s at 300 V (1200 V cm^{-1}) drive (see Fig. 3C and S1, ESI†). Protein patterning required just 100 pg of biotinylated c100p protein. After patterning the capture protein region, unbound biotinylated c100p antigen was electrophoresed out of the microchannel by applying a reverse polarity electric field. The process was repeated to create a barcode pattern of capture protein and spacer regions.

Patterning fidelity of capture protein

To assess both the magnitude and sources of barcode fabrication variability, we quantified band axial length and captured antigen mass (*via* AUC) for fluorescently labelled antigen. Here, we assessed: (i) triplicate channels loading from the same reservoir to understand channel-to-channel fabrication variation and (ii) channels across different chips to understand chip-to-chip fabrication variation. For channel-to-

channel variation, we compared a total of 9 different channels (sets of 3 connected to each of 3 reservoirs) patterned with c100p antigen under otherwise identical conditions. On each triplicate channel set, we observed between 3% and 8% standard deviation in the length of the patterned bands for bands patterned with a nominal $\sim 450\ \mu\text{m}$ axial extent as desired for the HCV assay ($n = 9$).

For the c100p patterning in these channels, we observed a standard deviation of between 3% and 6% in the AUC of the fluorescence signal from the immobilized c100p, again under otherwise identical conditions. Small observed differences in each metric are attributed to the batch fabrication approach employed: each triplicate channel set is fabricated from the same gel precursor solution with all channels exposed to the same UV conditions. Further, each triplicate channel set is concurrently loaded, during patterning, from the same sample reservoir (*i.e.*, sample solution).

In assessing chip-to-chip fabrication variation, we measured the amount of c100p protein patterned in the middle channel (2nd of each triplicate channel set) using the same approach. Although small, we opted to compare only the middle channels (for each triplicate loading from a single reservoir) to eliminate channel-to-channel variability discussed above. In this analysis, we observed a 3% standard deviation in the length of the patterned c100p band ($n = 3$, average length was $487\ \mu\text{m}$).

The role of Da in control of interfaces between barcode regions

To optimize and understand the interplay of electromigration and binding reactions – we assessed the final patterned capture protein distributions across a range of Da numbers (Fig. 3C, D). The sharpness of the patterned front was examined using the width (4σ) of the first derivative of the axial concentration distribution (Fig. 3C). To span a range of Da numbers (from 183 to 2.6), we varied the applied electric field ($E = 17$ to $1200\ \text{V cm}^{-1}$), with all other conditions held constant. We assume $L = 35\ \mu\text{m}$, $b_m = 16\ \mu\text{M}$, and $k_{on} = 3 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$.⁴² We noted a Da number associated dependence of both the axial and the transverse antigen concentration distributions, as discussed in this section.

In the $Da > 10$ regime ($E < 140\ \text{V cm}^{-1}$), where reaction kinetics are expected to be fast compared to electromigration, we observed a sharp boundary at the leading edge of the concentration front. We next explored the $10 > Da > 1$ transition regime ($1200\ \text{V cm}^{-1} > E > 140\ \text{V cm}^{-1}$) where the transport time becomes comparable to the reaction time. In this transition regime, the biotinylated c100p concentration front exhibited a broader axial concentration distribution than the sharp fronts observed in the $Da > 10$ region. In the $Da \sim 2.6$ regime, under the highest electric field loading condition ($1200\ \text{V cm}^{-1}$), the width of the concentration front was expanded to almost $0.3\ \text{mm}$. With Da slightly larger than 1, the reaction was anticipated to exhibit mass transport limited behaviour. Put another way, for this system, the time scale of the capture reaction ($t_r = k_{on}^{-1} \cdot b_m^{-1}$) is $0.02\ \text{s}$. Under a $50\ \text{V cm}^{-1}$ loading condition ($Da \sim 60$), a biotinylated c100p molecule can proceed only $0.6\ \mu\text{m}$ into the streptavidin-PA gel before being immobilized whereas at $1200\ \text{V cm}^{-1}$ ($Da \sim 2.6$) the same molecule should be able to migrate $15\ \mu\text{m}$ before

immobilization. As a trend, we observed a width that remains constant ($34 \pm 2\ \mu\text{m}$) for $Da > 10$ ($E < 140\ \text{V cm}^{-1}$) and increases with decreasing Da number.

In addition to (axially) broader leading edge behaviour, a transverse parabolic profile was observed with decreasing Da (*i.e.*, increasing electric field) as evident in Fig. 3C. While microfluidic systems dissipate Joule heating effectively due to favourable surface area to volume ratios, at high electric fields heat generation can raise the temperature and generate temperature gradients in the channel.^{43,44}

Inclusion of a 30 to 60 s incubation step ($E = 0\ \text{V cm}^{-1}$, no electromigration) during patterning of region (ii) enhanced biotinylated capture protein immobilization in the streptavidin-PA gel at the leading edge of the concentration front. During the incubation period, unbound biotinylated c100p in region (ii) diffused into region (iii), leading to a slow but sharp expansion of the interface according to a competition between reaction and diffusion. For high diffusivity species including biotin and biotinylated c100p (2 kDa), expansion of the leading edge resulted in extension of the patterned region by $\sim 50\ \mu\text{m}$ after 1 min of incubation.

Validating the selectivity of the barcode immunoassay

Having established the capacity to pattern capture proteins and spacer regions in a single channel with just one inlet and outlet, we next applied the approach to develop a barcode assay for human serum antibodies against three HCV antigens (core, c100p and NS3).

To yield a selective assay, each immobilized antigen region must specifically interact with the cognate antibody target, yet yield minimal cross-reactivity or nonspecific interaction with off-target antibodies. To understand the potential for cross-reactivity, we characterized two specific aspects of the assay: the cross-reactivity of our reagents and cross-reactivity inherent to the barcode format.

First, we investigated the possibility of nonspecific interactions between the reagents. To do this, we immobilized each capture protein in one of three different channels. Each immobilized capture protein was challenged by each of the three antibody targets individually (9 total channels). Fig. 4A shows the antibody signal in three channels, each housing a region of immobilized c100p capture protein. One of the target antibodies was electrophoretically loaded into each channel: $1\ \mu\text{g ml}^{-1}$ NS3, c100p, core (AF568).

As reported in Fig. 4A, we observed negligible signal from off-target antibodies (NS3, core) but did observe notable signal from the c100p antibody (SNR = 70). (This data shows non-uniform antibody immobilization across the target binding region, discussed in the next section.) Similarly, all three antibodies were loaded against channels individually patterned with NS3 and core capture proteins. Fig. 4B shows c100p and NS3 capture proteins reporting negligible off-target cross-reactivity. Core antigen, however, exhibited some non-specific capture of c100p (SNR = 38) and NS3 (SNR = 12) antibodies along with the expected core antibody capture (SNR > 200). Previous studies have shown that – under specific buffer conditions – core antigen exhibits nonspecific interactions with generic human immunoglobulin G (IgG) from the Fc γ fragment.^{45,46}

Fig. 4 Cross-reactivity and immobilized antigen purity. (A) HCV c100p was immobilized in 3 distinct channels and HCV NS3, c100p and core human antibodies (AF568 labeled) (6.65 nM , $1 \mu\text{g ml}^{-1}$) were injected. Only c100p antibodies were captured and produced signal. (B) Cross-reactivity matrix for three HCV antibody antigen pairs. Core antigen demonstrated some nonspecific capture as expected^{45,46} whereas NS3 and c100p pairs did not exhibit any cross-reactivity. Antibody signals were normalized to the maximum antibody signal for each assay. (C) NS3 and c100p antigens were immobilized in the same channel respectively. NS3 antibody injected into the channel goes through c100p antigen region without capture and gets captured only in NS3 region. C100p antibody injected to the same channel gets captured in the c100p region. (D) Same as part (C) with order of immobilized antigens and antibody injection order reversed, where no c100p antibody capture is observed at the NS3 antigen region. In summary, each antibody is captured in their corresponding antigen region, regardless of the order of immobilization.

Second, we studied a source of cross-reactivity inherent to the single channel barcode assay format; namely, the possibility of patterning more than one capture protein in each region. Based on the patterning principle, channel regions closest to the inlet will be patterned with capture protein first, necessitating that antigen used for patterning downstream regions does not “co-pattern” the already patterned near-inlet regions. In other words, new capture proteins must electromigrate through all previously patterned regions without becoming immobilized. Specificity in patterning is clearly necessary to allow the patterning of multiple capture proteins in a single inlet/single outlet channel.

To test patterning specificity, we immobilized first NS3 and then c100p antigens in a single channel (Fig. 4C). As such, NS3 was located closest to the channel inlet. After patterning of this multiplexed capture protein assay, NS3 Ab was loaded into the channel from the right. NS3 antibody capture ($\text{SNR} = 30$) was observed co-localized with NS3 suggesting that all streptavidin binding sites in the c100p region were saturated with either biotinylated c100p or biotin prior to assay commencement. Given this observation, we subsequently loaded c100p antibodies to the channel to confirm the presence and location of

the c100p region. In this c100p test, antibody capture is observed in the c100p region only ($\text{SNR} = 145$).

To further investigate the potential for unwanted co-patterning of capture proteins, we repeated the assay (in a new channel) but reversed the order of immobilization of the capture proteins as well as the order of the antibody load. When c100p antibodies were loaded, the c100p antibodies passed through the NS3 region with negligible interaction, yet the c100p antibodies did bind in the c100p region ($\text{SNR} = 172$). Similarly, we noted no off-target binding behaviour for the NS3 antibodies with capture localized to the NS3 region ($\text{SNR} = 41$). Thus, no nonspecific capture was observed supporting the hypothesis that patterning of single, unique capture proteins in spatially distinct regions of a single straight microchannel is possible.

Different antibody signals were observed for the NS3 and c100p readouts (Fig. 4C, D). Such differences in readout behaviour are not unexpected and may arise from a variety of sources. Specific differences may arise from: 1) Residual free biotin from the biotinylation reaction that may reduce the antigen immobilization efficiency. If unbound biotin was in the solution, some sites would be filled by the free biotin. This effect would become pronounced in cases where the protein mobility was low compared to the biotin mobility. A lower immobilized antigen concentration would lead to lower and more dispersed antibody capture in a given antigen band. 2) Intrinsic differences in binding affinity between different antibody antigen pairs may also contribute to lower or dispersed antibody signal. Rather than capturing the antibody mostly at the region entrance (e.g., c100p case), a lower affinity may lead to a disperse capture spread over the entire capture region (e.g., NS3 case). 3) Even if affinity is similar for antigen antibody pairs, biotin or fluorophore conjugation may block the active antigen epitopes leading to a reduced apparent affinity. 4) c100p is a 2 kDa peptide whereas the molecular mass of NS3 is 45 kDa. The relatively large size of NS3 can trigger steric hindrance during immobilization in the streptavidin sites or during the target antibody-antigen interaction. Nevertheless, for all antigen antibody pairs tested, target antibody detection was possible across a clinically relevant range (ng ml^{-1} – $\mu\text{g ml}^{-1}$).

Maximizing antibody detection with the barcode immunoassay

Antibody detection depends, in part, on the amount of antibody delivered to the capture protein region and the capture efficiency of that region. On the one hand, increasing the applied electric field strength will increase the total mass of target antibody probed by the capture protein over a set duration. On the other hand, depending on the Da regime, increasing the electric field may diminish capture efficiencies. To assess this interplay, we characterized antibody detection over a set 10 min long detection step for a range of electric field conditions. As with the patterning protocol, the detection of antibodies in the barcode immunoassay depends on both transport and reaction, now reaction in the form of antigen-antibody binding.

Fig. 5A reports antibody detection signal after 10 min loading of fluorescently labeled c100p antibody (AF-568

Fig. 5 Optimization of the antibody detection conditions. (A) Fluorescence micrographs of antibody introduction show detection is dependent on Da number. Solutions of AF-568 labelled c100p antibodies (13.5 nM) were electrophoresed into channels housing immobilized biotinylated c100p antigen using a range of voltages ($10\text{--}450 \text{ V}$, 10 min). (B) Endpoint fluorescence readout from labelled antibody for assays in (A). (C) Dose response curve for the unlabelled HCV-c100p human antibodies spiked in 2% human serum. Sandwich detection via AF-568 labeled goat antihuman antibodies. Lower limit of detection is 25 ng ml^{-1} or 165 pM with $\text{SNR} = 10$.

labeled $1 \mu\text{g ml}^{-1}$ 6.5 nM) across a range of applied electric fields. Here, c100p capture protein was immobilized in 12 different channels ($1 \mu\text{A}$, 9 V). Antibody signal increased with increasing electric field until 500 V cm^{-1} (125 V , $Da \sim 6.7$), as shown in Fig. 5B. In this regime reaction is mass transport limited such that signal is restricted by the rate of antibody delivered to the antigen sites. Put another way, nearly all antibody mass delivered to the binding sites is anticipated to be captured. For our estimated parameters ($L = 100 \mu\text{m}$, $b_m = 16 \mu\text{M}$, $k_{on} = 2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $\mu_i = 1 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$), the Da number remains larger than 10 when the $E < 320 \text{ V cm}^{-1}$. With $E > 320 \text{ V cm}^{-1}$ the operational regime spans $10 > Da > 1$, such that while the antibody delivery increases, the decline in the capture efficiency limits the total antibody capture. In this regime, reaction time becomes comparable to the time spent in the capture region. As an additional consideration, when the loading time exceeds $\sim 10 \text{ min}$, antibodies captured in the early stage of the detection stage may dissociate ($k_{off} \sim 10^{-3}\text{--}10^{-4} \text{ s}^{-1}$) from the capture protein and electromigrate down the channel under high field. Nevertheless, even at 1800 V cm^{-1} at 450 V drive, appreciable signal ($\text{SNR} > 1000$) was obtained. At this high voltage, gel deformation was observed (Fig. S3, ESI†). In summary, even at high fields, beyond the operation limit of the device, efficient antibody capture was observed.

We attribute the efficient antibody capture observed in Fig. 5B to utilization of directed electromigration of antibody target through a 3D gel matrix conjugated with a high density of antigen capture sites. Firstly, the gel matrix can support 2–3 orders of magnitude more capture reagent per given length of a microchannel section compared to channel walls functionalized with capture protein. Second, diffusion distances between antibody target and antigens immobilized in the 3D gel are notably reduced,³⁶ compared to channel wall functionalization approaches. Highly efficient antibody capture is validated by the non-uniform antibody signal across the target binding region shown in Fig. 4A, 4C and 5A. Delivered from the right direction, antibodies entering to the antigen regions

immediately get captured at the beginning of antigen regions leading to a signal peak towards the edge of the antigen regions.

Lastly, we sought to assess chip-to-chip variability in antibody detection. To accomplish this, we loaded fluorescently labelled antibody ($2 \mu\text{g ml}^{-1}$ AF568-labelled c100p antibodies) in three different channels on three different chips with all other conditions identical. We measured the AUC of the captured c100p antibody peak and observed a standard deviation of 26.6% ($n = 3$, range 115–192). We compare the performance of this prototype microfluidic barcode assay to that of a commercial strip immunoblot assay for reactive HCV antigens.⁴⁷ In this comparative study, the commercial immunoblot was performed multiple times on a single specimen (CHIRON RIBA HCV Processor System). The commercial system yielded a precision of $<16\%$ (range, 7 to 15.9%) for within-run and run-to-run testing.

Two primary sources of variability in fabrication of the microfluidic prototype assay were identified: (i) gel uniformity at the inlet of the patterned microchannels and (ii) uniformity of UV intensity during gel photopatterning. Firstly, imaging of antibody loading uniformity across the transverse channel dimension suggests that the presence of polymerized gel at the channel entrance (created in the reservoir during fabrication) contributes to variability during antibody loading. Specifically, channel entrance regions with visible gel debris may exclude introduction of large species (*i.e.*, antibodies) into the c100p patterned channel. A $\sim 10\%$ variation in transverse antibody concentration after capture on the patterned c100p was observed ($n = 3$, range 9.7% to 12.8%). Careful control of the polymerized gel interface in the entrance region – by either mask-based photo-polymerization or use of oxygen inhibition during chemical polymerization – has been shown to affect electrophoretic antibody loading.^{48,49} Secondly, prototype gel fabrication was accomplished using a lamp-based UV exposure system (UVP B100-AP), which provided uncollimated UV light over a small surface area ($\sim 3 \text{ cm} \times 3 \text{ cm}$), suitable for small batch chip fabrication. We envision a larger format UV

exposure system (e.g., mask aligner) as one potential avenue for addressing both sources of assay variability.

Barcode immunoassay validated for serological analyses

As shown in Fig. 5C, we applied the microfluidic barcode immunoassay to serological detection of human antibodies against a single HCV antigen: c100p. To test the microfluidic assay on a complex sample, we spiked unlabelled human antibodies against HCV-c100p in 2% healthy human serum (diluted 50-fold in TG buffer). 1%–2% is the typical serum dilution range used in Western blot and HCV-RIBA.¹⁰ A sandwich detection format was used, with fluorescently labelled (AF-568) anti-human goat antibody as the readout. Total assay runtime was 30 min with triplicate assays performed. All assay steps were performed electrophoretically (see Table S2, ESI†).

Fig. 5C reports the dose response curve from clinically relevant titres (5 ng ml^{-1} – $10 \mu\text{g ml}^{-1}$ in 2% human serum). Detectable signal was obtained down to 5 ng ml^{-1} (33 pM). The lower limit of detection (LOD) was determined to be 25 ng ml^{-1} (165 pM) where SNR = 10. Spanning an almost 3 orders of magnitude concentration range, the linear dynamic range of the assay was 33 pM–13.5 nM (5 ng ml^{-1} – $2 \mu\text{g ml}^{-1}$). Considering our observed 26.6% variability in the signal measurements, we estimate 16 discrete concentration levels spanning this dynamic range (i.e., binning). Compared to the four level qualitative binning readout provided by the current RIBA gold-standard assay, our prototype barcode assay yields both a larger dynamic range and quantitative readouts spanning a wider range of concentration levels.

These results suggest that the fluorescence-based microfluidic barcode assay is competitive with RIBA, while RIBA utilizes HRP based enzymatic signal amplification. Incorporation of enzymatic amplification to the microfluidic barcode assay presented could improve the LOD further. The microfluidic barcode assay required a 10 min antibody detection step, whereas RIBA prescribes a 4–8 h step. The assay was completed on 6 microliters of diluted sample, corresponding to 120 nanoliters of starting patient serum.

To further mature the microfluidic barcode assay, we developed a multiplexed serological assay for HIV and HCV antibodies. Three antigens and interleaving biotin spacers were patterned: protein L (loading control), HCV-c100p and HIV-p24. The protein L region was included to capture serum IgG and yield signal in cases where serum was successfully loaded. As a model system, we spiked $2 \mu\text{g ml}^{-1}$ of HCV-c100p (human) and HIV-p24 (mouse) antibodies in 2% human serum diluted in TG buffer. Detection antibody cocktail consisted of AF-568 labelled anti-human and anti-mouse IgG. Both the sample and secondary antibody loading conditions were optimized to ensure that the bulk concentration of reagents reached the channel outlet. Since the channel is short (only 2.5 mm) under the given operating conditions proteins migrate from one end to the other end in less than 30 s. Fig. 6 reports fluorescence micrographs and corresponding background subtracted fluorescence intensity profiles of two microchannels, labelled Ch1 and Ch2. Channel 1 assayed HCV-HIV antibody cocktail in 2% serum and channel 2 assayed 2% human serum without target antibodies. In channel 1, the

Fig. 6 Multiplexed microfluidic barcode assay for detection of serum antibodies to HIV and HCV. Top panel shows fluorescence micrographs of the Protein L, HCV-c100p and HIV-p24 antigen distribution in Channel 1. Channel 1 was assayed against the 2% serum sample spiked with $2 \mu\text{g ml}^{-1}$ (13.5 nM) HCV-c100p human and HIV-p24 mouse antibodies. A similarly patterned Channel 2 was assayed against 2% serum as a control. Detection used a cocktail of AF-568 labelled anti-human anti-mouse antibodies. Protein L regions exhibited saturated signal in both channels which confirmed the capture of highly available IgG in the serum as anticipated. HCV-c100p and HIV-p24 antibodies produced high signal in Channel 1 as shown in the fluorescence intensity profiles, which are background subtracted for comparison. Exposure time for the micrographs was 100 ms.

c100p region captured HCV-c100p antibodies and HIV-p24 region captured HIV-p24 antibodies. In both channels, the protein L region successfully detected IgG indicating successful serum loading.

Conclusions

As a basis for rapid, low-resource confirmatory diagnostics, we explore a simple single straight microchannel housing a multiplexed heterogeneous immunoassay. Key contributions centre on assay design, assay fabrication, and assay operational specifications. In design, we sought to use a ‘single inlet, single outlet’ microchannel to simplify device operation. To further simplify operation, we utilized a fully electrophoretic approach – no pumps or valves to support bulk fluid flow. We see the potential for such systems used in conjunction with a reader and disposables paradigm common in clinical laboratory assays. Further the possibility of integrating the single straight channel with a portable reader, such as a modified cellular phone, may prove powerful for specific applications.

In assay fabrication, we introduce a new electrophoretic patterning strategy that sees microchannel-filling streptavidin-decorated polyacrylamide gels as immobilization scaffolds. Sequential introduction of either free biotin or biotinylated capture proteins yields a “spacer-antigen-spacer” barcode pattern in the channel. To understand and ultimately optimize the fabrication process, we studied the patterning process across a wide range of Da numbers. We further characterized

antibody-detection – this time considering not the streptavidin-biotin reaction, but an antigen-antibody interaction – across a range of Da numbers. The empirical analyses inform both device fabrication as well as final assay operation.

In assay operational specifications, the format is purely electronic (single electrode pair) with electrophoresis conducted at low applied voltages. Low voltage and low power operation eliminates dependence on high voltage power supplies, pumps, syringes, or valves—making the device appropriate for near-patient operation. While available electrophoresis high voltage power supplies were used for this exploratory engineering study, the length of the barcode assay channel can be reduced, thus reducing the applied electric potential, making the format potentially suitable for operation with either a commercial battery or said battery and a voltage conditioning circuit. We validate the multiplexed microfluidic barcode assay for detection of immunoglobulin specific for HCV-c100p, HCV-NS3 and HIV-p24 antigens. The assay completes in 30 min with an analytical sensitivity comparable to the 6+ hour RIBA 3.0 assay. Performance advances are attributable to use of directed electromigration through functionalized 3D polyacrylamide gels.

Near-term directions include integration of the single channel microfluidic barcode assay with standalone electronics to yield an automated and portable confirmatory diagnostic platform. Given the run time and sensitivity of the assay, the microfluidic barcode assay could be a candidate to supplant laborious and time consuming confirmatory assays (e.g., RIBA). More broadly and longer term, a massively multiplexed version of the platform may be appropriate for screens used at blood banks and in pharmaceutical questions where high throughput (rapid and multiplexed) readouts are sought.

Acknowledgements

Authors thank Dr William Andrews, Dr Michelle Spatara, Dr Sejal Hall and Dr Eric Whitters (Novartis Diagnostics) and members of the Herr Lab for fruitful discussions. Financial support and HCV antibody and antigen reagents were provided from Novartis AG.

References

- 1 CDC, *Implementation of Routine HIV Testing in Health Care Settings: Issues for Community Health Centers*, 2011.
- 2 CDC, *Recommendations for Prevention and Control of Hepatitis C Virus (HCV) Infection and HCV-Related Chronic Disease*, in *MMWR*, 1998.
- 3 R. Chou, E. C. Clark and M. Helfand, *Ann. Intern. Med.*, 2004, **140**(6), 465–79.
- 4 J. H. Jou and A. J. Muir, *Ann. Intern. Med.*, 2008, **148**(11), ITC6-1–ITC6-16.
- 5 APHL, *Viral Hepatitis testing 2009 APHL survey report*, 2010.
- 6 F. Negro and A. Alberti, *Liver Int.*, 2011, **31**, 1–3.
- 7 P. Yager, G. J. Domingo and J. Gerdes, *Annu. Rev. Biomed. Eng.*, 2008, **10**, 107–44.
- 8 W. N. Burnette, *Anal. Biochem.*, 1981, **112**(2), 195–203.
- 9 R. J. Fido, A. S. Tatham and P. R. Shewry, *Methods Mol. Biol.*, 1995, **49**, 423–37.
- 10 www.fda.gov. Chiron RIBA HCV 3.0 Strip Immunoblot Assay, *Product Information, Package Insert*.
- 11 A. H. C. Ng, U. Uddayasankar and A. R. Wheeler, *Anal. Bioanal. Chem.*, 2010, **397**(3), 991–1007.
- 12 A. Bange, H. B. Halsall and W. R. Heineman, *Biosens. Bioelectron.*, 2005, **20**(12), 2488–2503.
- 13 X. Wen, H. He and L. J. Lee, *J. Immunol. Methods*, 2009, **350**(1–2), 97–105.
- 14 C. T. Lim and Y. Zhang, *Biosens. Bioelectron.*, 2007, **22**(7), 1197–204.
- 15 V. N. Morozov, *et al.*, *J. Am. Chem. Soc.*, 2007, **129**(42), 12628.
- 16 M. He and A. E. Herr, *Nat. Protoc.*, 2010, **5**(11), 1844–56.
- 17 T. Gervais and K. F. Jensen, *Chem. Eng. Sci.*, 2006, **61**(4), 1102–1121.
- 18 T. M. Squires, R. J. Messinger and S. R. Manalis, *Nat. Biotechnol.*, 2008, **26**(4), 417–26.
- 19 L. J. Lee, *et al.*, *Adv. Clin. Chem.*, 2006, **42**, 255–95.
- 20 H. Jiang, X. A. Weng and D. Q. Li, *Microfluid. Nanofluid.*, 2011, **10**(5), 941–964.
- 21 W. C. Yang, *et al.*, *Lab Chip*, 2010, **10**(19), 2527–2533.
- 22 G. A. Messina, *et al.*, *Anal. Biochem.*, 2008, **380**(2), 262–267.
- 23 C. H. Zheng, *et al.*, *Lab Chip*, 2012, **12**(14), 2487–2490.
- 24 C. D. Chin, *et al.*, *Nat. Med.*, 2011, **17**(8), 1015–1019.
- 25 D. D. Popovic Petar, B. S. Rabin and M. R. Shurin, ed., *Immunoglobulin Titers and Immunoglobulin Subtypes, Measuring Immunity: Basic Science and Clinical Practice*, ed. A. W. T. Michael and T. Lotze, 2005, Academic Press.
- 26 L. J. Kricka, *Clinical Chemistry*, 2000, **46**(8), 1037–1038.
- 27 J. Tate and G. Ward, *Clin. Biochem. Rev.*, 2004, **25**(2), 105–20.
- 28 L. S. Song, *et al.*, *Biomed. Microdevices*, 2012, **14**(4), 631–640.
- 29 R. Fan, *et al.*, *Nat. Biotechnol.*, 2008, **26**(12), 1373–1378.
- 30 J. M. Nam, C. S. Thaxton and C. A. Mirkin, *Science*, 2003, **301**(5641), 1884–1886.
- 31 A. A. Apori and A. E. Herr, *Anal. Chem.*, 2011, **83**(7), 2691–2698.
- 32 P. Englebienne, *Immune and Receptor Assays in Theory and Practice*, 1999, CRC Press.
- 33 M. He and A. E. Herr, *Anal. Chem.*, 2009, **81**(19), 8177–84.
- 34 M. Rendl, *et al.*, *Langmuir*, 2011, **27**(10), 6116–6123.
- 35 A. Bromberg, *et al.*, *Anal. Chem.*, 2012, **84**(2), 963–970.
- 36 A. J. Hughes, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**(16), 5972–5977.
- 37 A. Bernard, B. Michel and E. Delamarche, *Anal. Chem.*, 2001, **73**(1), 8–12.
- 38 S. Q. Tia, *et al.*, *Anal. Chem.*, 2011, **83**(9), 3581–8.
- 39 B. J. Kirby, *et al.*, *Lab Chip*, 2003, **3**(1), 5–10.
- 40 *The Benthic boundary layer: transport processes and biogeochemistry*, ed. B. P. Boudreau and B. B. Jorgensen, 2001, Oxford University Press.
- 41 E. L. Cussler, *Diffusion: mass transfer in fluid systems*, 3rd edn, 2009, Cambridge, New York: Cambridge University Press, xvii, p. 631.
- 42 M. Srisa-Art, *et al.*, *Anal. Chem.*, 2008, **80**(18), 7063–7.

- 43 E. Grushka, R. M. McCormick and J. J. Kirkland, *Anal. Chem.*, 1989, **61**(3), 241–246.
- 44 A. E. Jones and E. Grushka, *J. Chromatogr., A*, 1989, **466**, 219–225.
- 45 P. Maillard, *et al.*, *J. Biol. Chem.*, 2004, **279**(4), 2430–7.
- 46 A. M. Namboodiri, *et al.*, *Mol. Immunol.*, 2007, **44**(15), 3805–8.
- 47 P. Martin, *et al.*, *J. Clin. Microbiol.*, 1998, **36**(2), 387–90.
- 48 A. J. Hughes and A. E. Herr, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**(52), 21450–5.
- 49 A. J. Hughes, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**(16), 5972–5977.