

Bead-based immunoassays using a micro-chip flow cytometer†

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Received 22nd January 2007, Accepted 17th May 2007

First published as an Advance Article on the web 14th June 2007

DOI: 10.1039/b707507n

A microfabricated flow cytometer has been developed for the analysis of micron-sized polymer beads onto which fluorescently labelled proteins have been immobilised. Fluorescence measurements were made on the beads as they flowed through the chip. Binding of antibodies to surface-immobilised antigens was quantitatively assayed using the device. Particles were focused through a detection zone in the centre of the flow channel using negative dielectrophoresis. Impedance measurements of the particles (at 703 kHz) were used to determine particle size and to trigger capture of the fluorescence signal. Antibody binding was measured by fluorescence at single and dual excitation wavelengths (532 nm and 633 nm). Fluorescence compensation techniques were implemented to correct for spectral overspill between optical detection channels. The data from the microfabricated flow cytometer was shown to be comparable to that of a commercial flow cytometer (BD-FACSAria).

Introduction

Polymeric micro-particles have been used as solid supports in a range of surface binding assays for many years, and examples of bead-based assays include: immunoassays,¹ DNA hybridisation,² protein–protein binding³ and enzyme assays.⁴ There are many practical reasons for preferring bead-platforms for assays over solution-based methods or indeed other types of solid supports (e.g. protein microarrays): they provide an increased surface to volume ratio, reducing the reagent volumes required; beads move freely in solution so that the reaction kinetics are faster; immobilisation of biomolecules on the particle surface localises the chemistry to a defined point facilitating rapid analysis; a wide range of surface functionalised beads are commercially available and large numbers of beads can be rapidly analysed by flow cytometry.

Flow cytometry has been used as a detection method for bead-based immunoassays since the mid-1980s. Saunders *et al.*⁵ demonstrated a competitive binding assay using beads with a fluorescently labelled antigen competing with a non-labelled antigen for binding sites on unlabelled antibody-coated beads. The same group later demonstrated a flow cytometric assay for the antibiotic actinomycin D.⁶ This assay was based on the competitive binding of mithramycin (which fluoresces when bound to DNA) and actinomycin D (which does not fluoresce when bound to DNA) for sites on DNA-coated polymer beads. Bead-based sandwich assays targeting circulating immune complexes have been demonstrated by McHugh *et al.*⁷ A number of non-fluorescent bead-based techniques have also been developed, based on light scatter or extinction

measurements. These detect the particle aggregates that form when antigen-coated particles are incubated in a solution of free antibodies.^{8,9}

A number of studies have demonstrated the increased sensitivity, specificity and dynamic range obtainable with flow cytometric bead-based immunoassays over standard enzyme immunoassays.^{10,11} However, a major limitation of the bead assay is the complexity and expense of flow cytometer systems. To address this problem we are developing new micro-chip flow cytometers. In this paper we demonstrate that these devices are capable of performing bead-based immunoassays with comparable results to that of large-scale flow cytometers.

Miniaturisation is now a pervasive theme in bioanalysis, with the focus on development of systems that are cheap and capable of measuring ever smaller volumes of sample. Microfluidic devices are produced using techniques borrowed from the microelectronics industry and are revolutionising biochemical analysis systems.^{12,13} These micro-total analysis systems (μ TAS) or lab-on-a-chip (LOAC) devices are enabling technologies that have led to the development of small, cheap and often novel biochemical analysis devices.

The aim of this work is not to produce a chip-based system that duplicates the full functionality of currently available commercial FACS machines, but to demonstrate a flexible and easily configurable platform with many potential applications. Chip-based devices have a number of advantages over conventional flow cytometers. They can process extremely small sample volumes (tens of μ Ls) containing low numbers of particles while maintaining high statistical accuracy. They offer the possibility of integrating, within a single chip, sophisticated sample handling and detection modalities, allowing for the development of novel assays and measurement systems. One area of current research interest is the development of on-chip multiplexed assays using small numbers of size encoded beads.

Miniature flow cytometers were first demonstrated by Kamensky in the mid-1960s.¹⁴ Since then various microfluidic-based devices have been reported in the literature for

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† The HTML version of this article has been enhanced with colour images.

handling and analysing a variety of micro- and nano-particles. A number of microfabricated devices have been reported which either assay beads by flowing them through a detection volume,^{15–24} or immobilise them on-chip as a packed bed through which a sample flows.²⁵

A small sample of beads is introduced into the device, and these flow under external applied pressure through a detection region in the chip. The device measures the fluorescence from the beads as they pass along a microfluidic channel at high speed. Negative dielectrophoresis (nDEP) is used to focus the particles into the detection zone, ensuring that all the particles pass through this region with a constant velocity and at a reproducible height.²⁶ The use of dielectrophoretic focussing rather than hydrodynamic focussing simplifies the construction and operation of the system. Precise 2-D hydrodynamic focussing is difficult to implement¹⁵ and requires complex fabrication for integration within LOAC devices.

In order to simplify the optical detection system, the scattered light is not measured, but rather the low frequency electrical impedance of the beads is measured. This is performed using micro-electrodes patterned on the upper and lower surfaces of the microfluidic channel, and is used to indicate particle size (complementary to the forward scatter (FSC) signal on a conventional flow cytometer). The impedance signal is also used as the trigger signal for data acquisition. Optical scattering is a method for accurate particle sizing, however the technique is difficult to implement on-chip. There are a number of applications where it would be advantageous to use impedance-based particle sizing rather than a more complex optical setup based on planar waveguides or external optical components.

The device has been used to assay an immunoreaction, based on the binding of fluorescently labelled anti-human IgG and anti-rabbit IgG molecules to human IgG and rabbit IgG coated polymer beads. Data from the micro flow cytometer was compared with data obtained from a commercial flow cytometer (BD-FACSaria).

Fig. 1(a) shows a schematic diagram of the chip illustrating the channel, the impedance detection electrodes and the fluorescence illumination and detection region. The DEP focussing electrodes (not shown in Fig. 1) are positioned upstream of the detection area. Further details of the DEP focussing can be found in a previous publication.²⁶ A photograph of the chip is shown in Fig. 1(b). Particles flow into the chip from the left and are carried through the device by the fluid. Electrodes on the upper and lower internal walls of the channel focus the particle stream so that all particles pass through the detection zone.²⁷ Two further pairs of electrodes form the impedance detection zone. The optical interrogation zone is positioned either between the impedance measurement electrodes or a few micrometres downstream. The electrical and optical signals are digitised and stored for analysis.

Materials and methods

Sample preparation

Recombinant protein A was purchased from Cambridge Bioscience (UK). Polyclonal human IgG (immunoglobulin G) and polyclonal rabbit IgG were obtained from Sigma (UK).

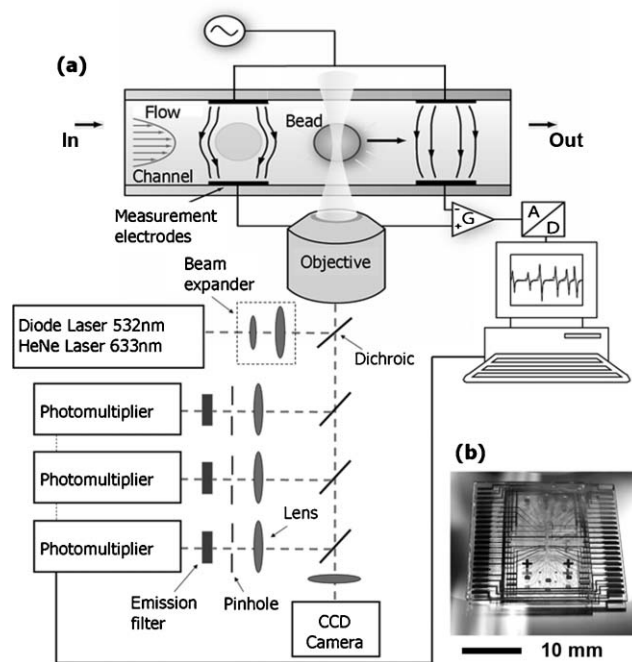


Fig. 1 (a) Schematic diagram of the micro flow cytometer system, showing the confocal-optical and electrical detection setup. Microelectrodes within the flow channel produce DEP-induced particle focussing. The electrodes (not shown) are positioned upstream from the impedance detection electrodes. (b) Photograph of an individual micro-chip (scale bar = 10 mm).

Cy3-labelled goat polyclonal anti-human IgG (anti-human IgG) and Cy5-labelled goat polyclonal anti-rabbit IgG (anti-rabbit IgG) were purchased from Abcam plc. (UK). GMA (glycidyl methacrylate) microspheres (5.6 μm diameter) were obtained from Bangs Laboratories (IN, USA). A BCA assay kit was obtained from Pierce (UK). DMF (*N,N*-diisopropylethylamine), EDC·HCl (*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride), succinic anhydride, TEA (triethylamine), Tween20 and other reagents were obtained from Acros (UK), Alfa Aesar (UK) and Fisher (UK).

Preparation of carboxy GMA

Amino GMA (100 mg, 0.53 μmol based on amino groups) was shaken with a solution of succinic anhydride (1 mg, 10 μmol) and TEA (1.4 μL , 10 μmol) in dry DMF (400 μL) at room temperature for 1.5 h. The reaction mixture was centrifuged (16 000 $\times g$), the supernatant was discarded and the coupling step repeated with fresh reagents for a further 1.5 h. The completeness of the reaction was monitored by a ninhydrin test. After a negative ninhydrin test,²⁸ the microspheres were washed with dry DMF (1 mL $\times$ 4) and MeOH (1 mL $\times$ 4), the supernatant was separated by centrifugation (16 000 $\times g$), and dried *in vacuo* overnight.

Preparation of protein A coated GMA

Carboxy GMA beads (3 mg, 16 nmol based on loss of amino group after succinylation) were shaken with a solution of protein A (10 μL , 50 mg mL⁻¹, 11.9 nmol) and EDC·HCl (9.2 mg, 48 μmol) in buffer A (100 mM imidazole, pH 7,

600 μL) at room temperature overnight. The microspheres were washed with buffer A (1 mL $\times$ 4) followed by buffer B (10 mM NaH_2PO_4 , 150 mM NaCl , 0.1% Tween20, pH 7.8, 1 mL $\times$ 4), the supernatant was separated by centrifugation (16 000 $\times g$), and the microspheres were stored in buffer B (1 mL). The amount of protein immobilised was determined by BCA assay.²⁹

Preparation of IgG conjugated GMA

A solution of human IgG (10 μL , 5.8 mg mL^{-1}) or rabbit IgG (10 μL , 5 mg mL^{-1}) in buffer B (190 μL) was added to protein A coated GMA beads (0.3 mg, 95 nmol g^{-1} based on protein after immobilisation) at room temperature for 1 h with gentle mixing. The microspheres were washed with buffer B (1 mL $\times$ 4), the supernatant was separated by centrifugation (16 000 $\times g$), and resuspended in the same buffer (200 μL).

Antibody assay

A solution of Cy3-labelled anti-human IgG (2 μL , 1 mg mL^{-1}) and Cy5-labelled anti-rabbit IgG (2 μL , 1 mg mL^{-1}) in buffer B (46 μL) was added to a mixture of human IgG and rabbit IgG-conjugated GMA beads (0.3 mg each) at room temperature for 1 h with gentle mixing. The microspheres were washed with buffer B (1 mL $\times$ 4), the supernatant was separated by centrifugation (16 000 $\times g$), and resuspended in the same buffer (200 μL).

Microfluidic flow cytometer

Microfluidic chip

The microfluidic chips were fabricated using photolithography and full wafer thermal bonding. The process was as follows: 500 μm thick 4" pyrex wafers (Schott, Switzerland) were cleaned in H_2SO_4 followed by rinsing in distilled water and dried under nitrogen. A double-layer resist was deposited using an EVG150 automatic spin coater (EVG, Germany). The wafers were dehydrated and a 0.45 μm thick layer of LOR5A (Microchem) photoresist was spun onto the wafers (4000 rpm) and baked (8 min at 183 $^\circ\text{C}$). This was followed by a 1 μm thick layer of S1813 photoresist (Shipley, 6000 rpm) baked at 115 $^\circ\text{C}$. The resist layer was photolithographically patterned (Karl Suss MA-6 Mask Aligner, Germany) and developed using Microposit MF-319 metal-ion-free developer (Shipley). 100 nm of Pt was evaporated onto the substrate (LAB-600H, Laybold Optics, Germany). The resist layer, together with unwanted metal was removed by ultrasonication in Microposit Remover 1165 (Shipley).

A thick photosensitive polyimide precursor (PI 2729, Dupont) was used to define the microfluidic channels. This was spun over the patterned electrodes at 4000 rpm. A preliminary adhesion promoter (VM-651, Dupont) was used to prevent edge detachment of the polyimide layer during development. A soft bake step, 5 min at 60 $^\circ\text{C}$ followed by a 5 min at 100 $^\circ\text{C}$, was performed prior to the layer exposure. Photolithography was done in hard contact mode and the exposure parameters were 40 s at 10 mW cm^{-2} (MA-6, Karl Suss). The polyimide development process was repeated three times in a cycle of three consecutive baths of 100% developer

(DE6180, Dupont), 50–50% developer and rinse mixture and 100% rinse (RI9180, Dupont), with 30 s in each bath while maintaining constant agitation. A final rinse was performed in fresh rinse solution and development was checked optically. Two identically processed wafers were then thermally bonded (SB-6, Karl Suss), under a tool pressure of 5 bar, ramping the temperature to 200 $^\circ\text{C}$ for a bake of 30 min in air and followed by 300 $^\circ\text{C}$ in N_2 for 1 h.

Individual chips were released from the wafers by dicing with a diamond saw (DAD321, Disco Corporation). Inlet and outlet holes were drilled (carbide tipped drills, Drill-Service Ltd) in the individual chips to allow fluidic access. The channel dimensions in the detection region were 20 μm wide by 20 μm high.

Electrical and fluidic interconnections to the chip were made using a custom built connection block, machined from PEEK polymer. Fluidic and electrical connections are made by clamping the chip within the block. Spring loaded electrical connectors (SAMTEC UK, Cumbernauld, UK) were used to make contact with the chip electrodes and miniature O-rings used to seal the fluidic connection between the block and chip. Microfluidic tubing and connectors were purchased from Lee Products Ltd. (Bucks, UK) and used to connect the PEEK block to the sample, waste and wash reservoirs. The connector block is mounted on a PCB which has the impedance detection circuitry and the DEP driving electronics. The entire assembly is mounted on an x-y-z stage above a microscope objective, allowing alignment of the chip and accurate positioning of the optical detection region within the microfluidic channel. The bead sample is introduced into the chip and flowed at a constant velocity through the device using a positive pressure.

Optical detection

The optical system (Fig. 1 and 2) was designed to measure fluorescence from single particles simultaneously at three different wavelength ranges (~ 585 nm, ~ 675 nm and >715 nm). Light from a 532 nm (20 mW solid state YAG) and a 633 nm (20 mW HeNe) laser were coupled into the channel using free-space optics. The laser beams were combined, passed through a beam expander and into an infinity corrected objective lens (Nikon x20, NA = 0.7) to form a diffraction limited spot in the centre of the channel. Fluorescent light was collected by the same objective lens. Dichroic mirrors and bandpass filters spectrally filtered the light before spatial filtering with lenses and pinholes (100 μm , Thorlabs) positioned in front of photomultiplier tubes (H7710-13, Hamamatsu). A power supply (C7169, Hamamatsu) was used to adjust the gain on the PMTs and the signals were conditioned and amplified with Hamamatsu amplifiers (C7319, Hamamatsu). The intensity of the light at the focal point of the objective was measured as 3.3 mW for the red laser and 600 μW for the green. Details of the dichroics and bandpass filters are shown in Fig. 2; all were purchased from Chroma Technologies (Rockingham, VT, USA).

Impedance detection

Details of the single micro-particle impedance detection system have been described previously^{16,23,24} and therefore will only

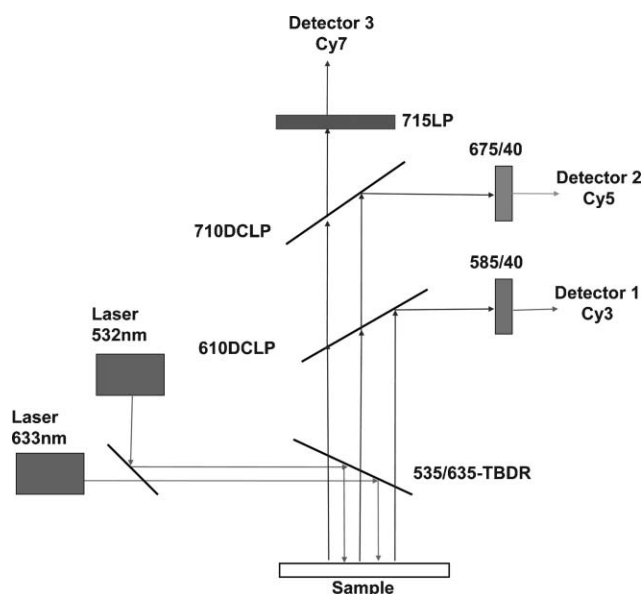


Fig. 2 Details of the optical detection system showing the various optical filters and dichroic mirrors. The setup allows dual laser excitation (532 nm and 633 nm) and detection of Cy3 (~ 585 nm), Cy5 (~ 675 nm) and Cy7 (> 715 nm).

be described in brief. One or more sinusoidal voltages at fixed frequencies (100 kHz–10 MHz, 0.5 Vpp (peak-to-peak voltage)) are applied to both pairs of detection electrodes. The electrodes and channel volumes between the electrode pairs form the two arms of a differential circuit. When a particle moves between the electrode pairs, a differential current flows in the system. The signal from the microfluidic chip is amplified with custom built electronics, and lock-in amplifiers (SR844, Stanford Research Instruments, USA) are used to detect the signals, one lock-in for each frequency. The output from the lock-in is a complex signal, consisting of the real component (in phase) and imaginary component (90° out of phase) of the electrical impedance at each particular frequency. The outputs from the lock-in amplifiers, and the photomultipliers are sampled at 100 kHz with a 16-bit A–D card (NI6034E, National Instruments) and the data captured and analysed with software written in MATLABTM (Mathworks Inc., Natick, MA, USA). The impedance data was used to measure the size of the beads and trigger the data acquisition. A peak detection algorithm was used to detect the fluorescence signals; data was processed and plotted on scatter plots for subsequent analysis.

Particle focussing

Focussing particles into a well-defined detection volume is an essential element of flow cytometry. In this device, dielectrophoresis was used to focus the particles. The micro-electrodes (on the top and bottom internal surfaces of the channel) ensured that all the particles were focussed as they flowed through the channel. The focussing electrodes are only 100 nm thick and do not perturb the fluid flow in any appreciable way. They are positioned upstream from the detection region. When an AC voltage is applied to these electrodes (10 Vpp at 8 MHz, anti-phase to diagonally opposite electrodes) particles are repelled

from the regions of high electric field (*i.e.* electrode edges) and are focussed into the central streamline of the channel.^{26,31} This technique ensures that all particles pass through the measurement region with a uniform velocity and trajectory.

Commercial flow cytometer

Classical flow cytometric analysis of the particles was carried out with a BD-FACSARIA (Becton Dickinson) equipped with two lasers: a 488 nm solid state (13 mW–20 mW, Coherent[®] SapphireTM), and a 633 nm HeNe (10 mW–20 mW, JDS UniphaseTM). FACSFlow sheath fluid (Becton Dickinson) was used and samples were flowed at a pressure of 70 psi through a 70 μ m nozzle. The instrument was controlled by a PC running FACSDiVa software (Becton Dickinson).

Side Scatter (SSC) and orange fluorescence (575/26 nm) were measured using laser excitation at 488 nm. Red fluorescence (660/20 nm) was measured with laser excitation at 633 nm.

Data analysis

Fluorescence compensation

Fluorescence compensation is a mathematical technique used to correct multiparametric (multi-colour) flow cytometric data for spectral overlap.^{32,33} Spectral overlap occurs when the emission spectrum from fluorescent dyes appear in more than one detection channel. The technique enables correction of spillover from the various fluorophores into each of the detector channels. In the commercial FACS machine that was used in this work (BD-FACSARIA), the lasers illuminate separate areas of the flow channel and the detectors are spatially offset so that spillover is minimised. In our device, both lasers are incident on the same spot, so that spectral spillover is possible.

In general, assuming that n different fluorophores are measured, and that all of these might contribute some level of fluorescence signal to each of the n detection channels (assume one channel for each fluorophore), then the measured signal in each channel is given by the following set of equations:³³

$$F(1) = k(11) \times f(1) + k(21) \times f(2) + \dots k(n1) \times f(n) \quad (1)$$

$$F(2) = k(12) \times f(1) + k(22) \times f(2) + \dots k(n2) \times f(n)$$

$$F(n) = k(1n) \times f(1) + k(2n) \times f(2) + \dots k(nn) \times f(n)$$

where $F(i)$ is the measured fluorescence in channel i and $f(i)$ is the actual amount of fluorescent molecule i present on the particle of interest. The spillover coefficients $k(ij)$ represent the ratio of the mean fluorescence of fluorophore i in channel j to the mean fluorescence of fluorophore i in channel i . $k(ij)$ can be calculated *ab initio* with detailed knowledge of the spectral properties of the various fluorophores, optical filters and illumination sources used in the system. However, it is simpler to determine the spillover coefficients empirically by running samples labelled with only a single fluorophore. In other words if we are compensating for fluorescent molecule i , then $f(j)$ will be zero for every j that is not equal to i . The coefficient $k(ij)$ is equal to the measured value $F(j)$ divided by the measured value $F(i)$; note $k(ij) = 1$ for $i = j$.

Once the coefficients $\mathbf{k}$ have been determined, the amount of each fluorescent molecule can be obtained by solving the above set of equations. In matrix algebra, these equations are written as:

$$\mathbf{F} = \mathbf{k} \times \mathbf{f} \quad (2)$$

Computationally, the coefficient matrix $\mathbf{k}$ is inverted, multiplied by the measured values $\mathbf{F}$, and the result $\mathbf{f}$ gives the concentration of each fluorescent species present. $\mathbf{f}$ is the set of compensated signals and the coefficients in the inverse matrix $\mathbf{k}'$ contains the compensation values.

$$\mathbf{f} = \mathbf{k}' \times \mathbf{F} \quad (3)$$

Where compensation is required for two fluorophores (*e.g.* Cy3 and Cy5) the calculation is relatively simple and can be made without the use of singly labelled particles, as done in this paper. However, as the number of fluorophores and detection channels increases, compensation becomes more complicated and calibration requires running a full series of singly labelled particles (one for each fluorophore used), then employing the above calculations to obtain the compensation values.

Results and discussion

Samples of human IgG and rabbit IgG-labelled beads were prepared as described above. The bead suspension was incubated with the fluorescently labelled Cy3-anti-human and Cy5-anti-rabbit antibodies for analysis (see Fig. 3).

The bead mixture was adjusted to $\sim 5 \times 10^5$ beads per mL by the addition of PBS (phosphate buffered saline, Sigma, UK) and then analysed on the micro flow cytometer. Individual particles passing through the chip were focussed by negative DEP into a narrow stream. As they passed through the detection zone their optical and low frequency electric properties were measured simultaneously. Fig. 4 shows typical data obtained from individual beads when analysed by the micro flow cytometer. Fig. 4 (a) and (b) show data streams when a single Cy5-anti-rabbit labelled bead and a single Cy3-anti-human labelled bead are detected. The bipolar signal is the impedance signal as the bead passes between the first and then the second of the electrode pairs. In this data, the signal applied to the detection electrodes was 0.5 Vpp at a frequency of 703 kHz. For these beads, and at this frequency, the in-phase component of the impedance signal can be considered to be proportional to the particle volume. The data capture and analysis software was adjusted so that triggering occurred over a narrow range of voltages from the impedance channel. This method rejects errors from smaller contaminating particles (*e.g.* debris) within the sample. The low-frequency impedance signal is in many ways similar to the FSC signal obtained in a conventional flow cytometer, giving a measure of particle size. Note that sizing particles using low-frequency impedance measurements provides a more accurate measure than the “small angle” or FSC measurement, due to the non-monotonic nature of the optical scatter signal.³⁰ Similarly, using impedance methods, particle aggregates (doublets and triplets) can be detected and excluded (or “gated out”) from the data.

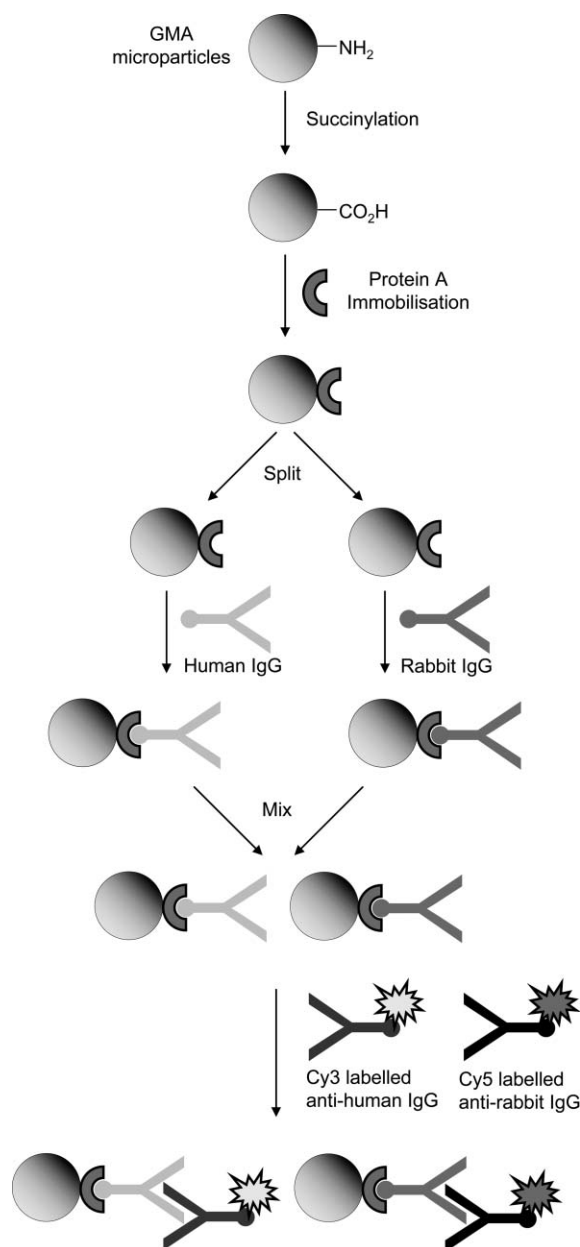


Fig. 3 Outline of the immunoassay based on the use of 5.63 μm GMA beads with fluorescent labelled antibodies to human (Cy3) and rabbit (Cy5) IgG. Coupling of the rabbit IgG and human IgG was carried out in separate tubes. The two populations were then mixed and the anti-human and anti-rabbit antibodies added. Samples were incubated and analysed using FACS and the micro flow cytometer.

In the micro flow cytometer, the impedance signal is measured in a zone approximately 30 μm upstream from the optical detection region, this ensures that any heat generated by the laser light does not interfere with the impedance measurements.³⁴ Typical traces from optical signals from the orange (585/40 nm) and red (675/40 nm) detection channels are shown in Fig. 4. In the Figure, the signal from the red channel has been offset by 0.05 V to allow for better visualisation of the data. Fig. 4 shows data obtained when only the 532 nm laser was used. The effect of spectral overlap can be seen in this data, with the Cy3-anti-human beads producing a signal in both the orange and red channels.

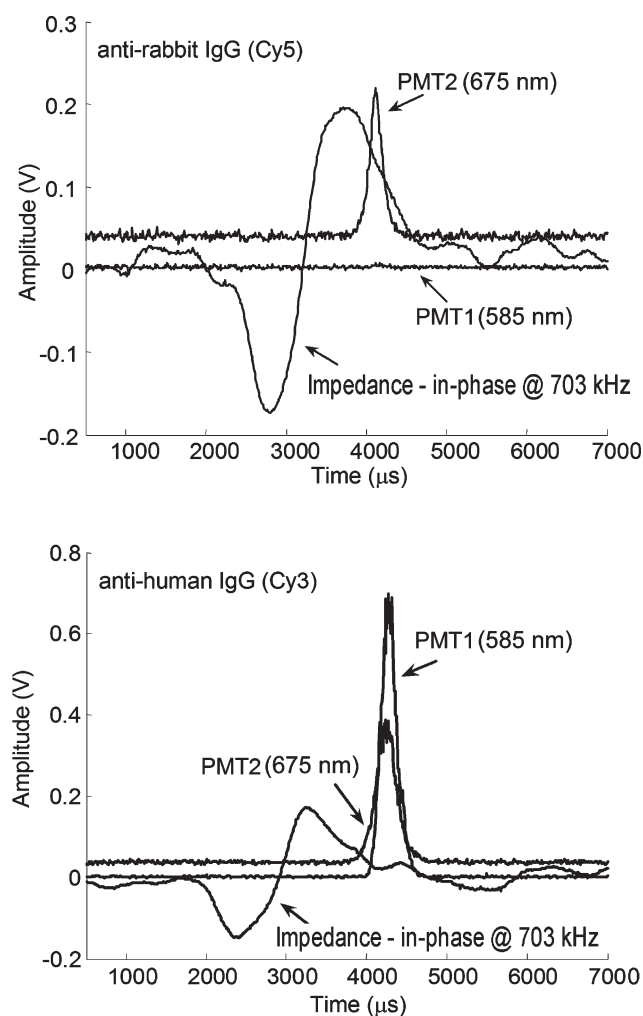


Fig. 4 Data from an individual bead detected in the micro flow cytometer. Orange (585/40 nm) and red (675/40 nm) fluorescence signals are shown along with the in-phase component of the impedance signal measured at 703 kHz. The red signal is offset by 0.05 V to allow better visualisation. (a) Data from an individual Cy5-anti-rabbit conjugated bead and (b) Cy3-anti-human bead. The experimental conditions under which this data was collected were; suspending medium conductivity = 1.6 Sm^{-1} , bead diameter = $5.63 \text{ }\mu\text{m}$, particle velocity = 10 mm s^{-1} .

Spectral overlap

Measurement of samples containing particles labelled with more than a single fluorophore (*e.g.* Cy3 and Cy5) can be difficult to analyse quantitatively due to spectral overlap from the multiple fluorophores in the emission spectra. Simultaneous illumination by more than one colour laser (532 nm and 633 nm) further complicates the analysis if the fluorophores' excitation spectra overlap and include both excitation wavelengths. In multiparametric flow cytometric analysis the light emitted by individual particles is simultaneously detected *via* a number of different optical channels, each with a well-defined spectral range. As described earlier, the BD-FACSAria data does not require fluorescence compensation because the two laser beams (488 nm and 633 nm) are spatially separated within the flow cell. Furthermore, for the dyes and wavelengths used in this work, each laser in the

BD-FACSAria system has its own dedicated, spatially separate detection channel and spectral overlap is less of an issue.

To simplify the optical system in the micro flow cytometer, both the 532 nm and 633 nm lasers illuminate the same volume, and all three detection channels collect light from the same point simultaneously. This design leads to correlations between the various detection channels. The filters used in the micro flow cytometer are shown in Fig. 2 and were designed to minimise spectral overlap. However, to quantify the data from the micro flow cytometer (and FACS systems in general) the correlations due to spectral overlap have to be removed. The optical measurements shown in Fig. 4 and 5 were obtained using single laser (532 nm) excitation.

The traces in Fig. 4 clearly show spectral overlap, indicating a strong correlation between the orange (585/40 nm) and red (675/40 nm) channels for the Cy3-anti-human labelled beads. This is due to the broad emission spectrum of the Cy3 fluorophore when excited by the 532 nm laser. No correlation is seen for the Cy5-anti-rabbit labelled beads as the emission spectrum of Cy5 is very weak in the orange (note it is also weak in the red).

Fig. 5 shows a scatter plot for a typical data set (~ 500 events) obtained from a sample of Cy3-anti-human and Cy5-anti-rabbit labelled beads analysed on the micro flow cytometer with dual 532 nm and 633 nm laser excitation. Fig. 5(a) shows the correlation from the Cy3 beads, measured in the orange (585/40 nm) and red (675/40 nm) channels.

The spectral compensation technique described by Bagwell and Adams³³ and detailed above was applied to this data in order to remove spectral overlap. Fig. 5(b) shows the data after compensation for spillover of Cy3 fluorescence into the red channel (compensation coefficient $k_{21} = 0.7$). The Cy5 emission in the orange channel is negligible and can therefore be ignored ($k_{12} = 0$).

Comparison with commercial FACS

The samples were also analysed using a BD-FACSAria and the data is shown in Fig. 6. In this case, the data was collected by triggering on the side scatter signal. The data from 5000 events is shown; no compensation is necessary because of the spatially separate excitation and detection channels. Both the compensated data from the micro flow cytometer (with dual laser excitation shown in Fig. 5(b)), and the BD-FACSAria data shown in Fig. 6 show excellent agreement. The small differences seen in the data are due to the fact that the BD-FACSAria excites and detects at different wavelengths; 488 nm compared with the 532 nm used in the micro flow cytometer.

With compensation, the scatter plots of fluorescence emission for each subpopulation are similar; beads can be clearly discriminated in both cases. For the data set shown in Fig. 5 (micro flow cytometer) the relative percentage of particles in regions (1) and (2) were 38.8% and 61.1%, respectively. When this population of particles was analysed using the BD-FACSAria, the relative percentages were 35.7% and 53.9% for R1 and R2. The discrepancy between the two systems is due to the inclusion of events in region R3 in the BD-FACSAria plot. The events in R3 are due to noise and small particles, such as protein aggregates or other debris in

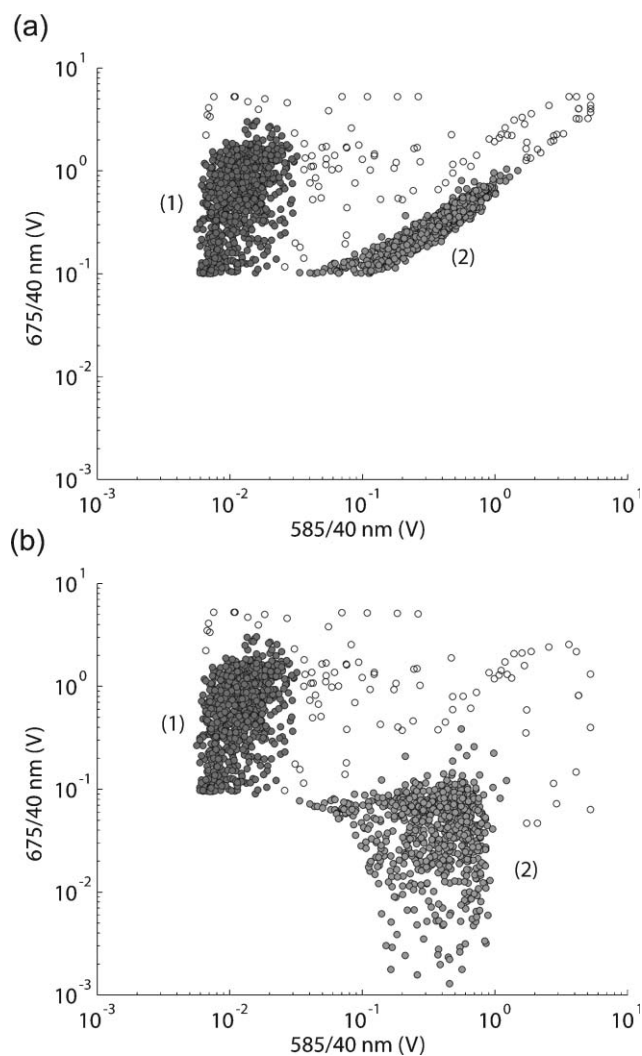


Fig. 5 Scatter plots showing the data obtained from micro flow cytometer analysis of the bead mixture using dual laser illumination (532 nm and 633 nm). (a) A clear correlation can be seen between the orange (585 nm) and red (675 nm) channels in the uncompensated data due to the large spectral overlap at these wavelengths from the Cy3 fluorophore. (b) The data with spectral compensation applied ($k_{21} = 0.7$ and $k_{12} = 0$).

the sample. These particles produce sufficient scattered light to trigger data acquisition, but are too small to trigger the micro flow cytometer using the impedance signal. Gating out the events in R3 (Fig. 6) and recalculating gives relative percentages of 40.2% and 59.1% for R1 and R2, respectively.

We have previously shown excellent agreement between results obtained using the micro flow cytometer and those obtained with the BD-FACSaria.^{24,26} The data shown in Fig. 4–6 were replicated for several different experimental runs, all showing excellent correlation between the micro flow cytometer and the commercial system (BD-FACSaria).

System sensitivity

In order to quantify the sensitivity of the micro flow cytometer system, we calculated the average number of fluorophore molecules on the surface of the beads. The total amount of

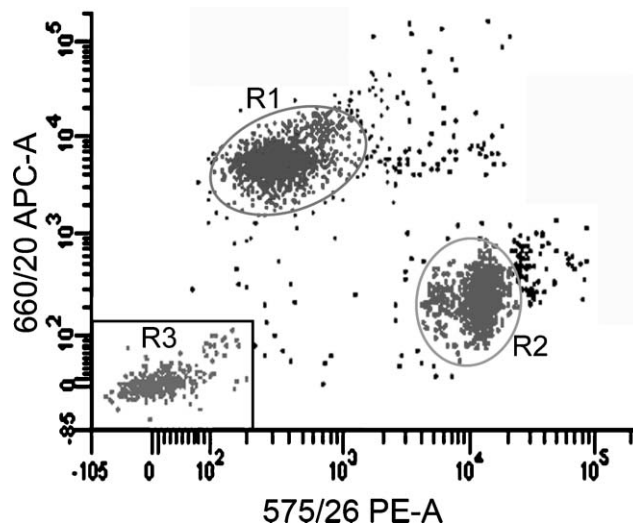


Fig. 6 Scatter plot showing data from the same sample shown in Fig. 5, analysed on the BD-FACSaria. R1 and R2 show the Cy5-anti-rabbit labelled beads and Cy3-anti-human beads respectively. R3 is noise due to protein aggregates and other debris in the sample and can be gated out.

protein A bound to the beads was determined from the BCA assay. For a sample of 0.3 mg of beads this was 28.8 pmol, giving 6.87×10^6 molecules of protein A per bead. Assuming that the protein A molecule has a cross-sectional area of $5 \text{ nm} \times 5 \text{ nm}$ this equates to approximately one (1.19) monolayer per bead. The amount of anti-human IgG was determined by titration to be 3.05 pmol for 0.3 mg of beads, equivalent to 0.73×10^6 IgG molecules per bead. Assuming the dimensions of the molecule to be $10 \text{ nm} \times 10 \text{ nm}$,²⁹ this is approximately 0.5 monolayers. This figure is lower presumably because steric hindrance limits the number of protein A molecules that bind IgG molecules. Each IgG molecule is on average conjugated to 9 Cy3 molecules (data provided by supplier), giving an average of 6.57×10^6 Cy3 molecules per bead. Particles with this level of fluorescence were easily detected by the micro flow cytometer at the flow velocities used in the experiment, $\sim 50 \text{ mm s}^{-1}$.

The sensitivity of the device was further evaluated using commercially available calibration beads (Molecular Probes, L-14819). These are sets of identically sized, $6 \mu\text{m}$ diameter latex beads with relative fluorescence intensities distributed on a log scale: 100%, 16.1%, 0.54%, 0.12% and 0.028%. The mean values of the fluorescence intensities for the bead populations were measured and found to be quantitatively similar on both the BD-FACSaria and the micro flow cytometer (data not shown). For the instrument settings used (PMT gain, laser intensities, flow velocities) the mean fluorescence measured from the protein-conjugated beads was 6.2 times greater than the lowest intensity calibration beads (0.028%) and the signal to noise ratio (SNR) of the 0.028% beads was 41.4. This translates to a lower limit of detection for the micro flow cytometer of $\sim 2.6 \times 10^4$ fluorescent molecules per bead (at the same laser power and flow rate, *etc*). The bandwidth (response time) of the optical detection system in the micro flow cytometer is sufficient to allow accurate measurement of

particle fluorescence at higher velocities than those used. However, at extremely high flow velocities the residence time of the beads in the detection zone will reduce the overall sensitivity. A further limitation of the current system is that at high flow rates the DEP focussing becomes less efficient and particles no longer pass through the centre of the optical detection zone.²⁶ This increases the coefficients of variation (CV) in the measured data (a similar increase in the CV is seen in the impedance measurements). Future development of the microfluidic chip geometry and improvements to the optical system (e.g. use of a higher NA objective lens) will afford higher rates of particle throughput and further increase the optical sensitivity of the instrument.

Measurements using size calibrated latex particles (data not shown) demonstrate that the impedance measurements provide accurate sizing of beads in the size range 2 µm–10 µm in diameter, with 1 µm resolution. The sensitivity is primarily related to the dimensions of the channel, which in the current device is 20 µm × 20 µm. Beads larger than 10 µm can block the channel, beads smaller than 2 µm diameter are too small for the present design of electronics to detect above the noise.

Conclusions

We have demonstrated a microfluidic device capable of rapid analysis of small volumes of fluorescently labelled polymer beads. The system was used to count and assay micron-sized polymer beads used as a solid support for a fluorescence immunoassay. DEP focussing was used to position particles as they flowed through the device, allowing accurate impedance-based sizing of the particles. The impedance signal was used to trigger data acquisition and capture of fluorescent data from individual beads as they passed through an optical interrogation zone immediately downstream from the impedance detection electrodes.

The performance of the micro flow cytometer was compared with a commercial system (BD-FACSAria). The data shows that the two systems are comparable, except that the micro flow cytometer has a lower throughput—100s of particles per minute compared with ~1000 particles per second typical in the BD-FACSAria. The effects of spectral overspill into the different optical detection channels of the micro flow cytometer were corrected. This compensated data was shown to be directly comparable to data from the BD-FACSAria.

Scaling up of the system to perform immunoassays, more complex than those demonstrated here, is relatively simple. To increase the number of possible targets under assay one can use two methods: increase the number of distinct fluorophores used in an experiment and/or use encoded micro-particles. Obviously, as the number of fluorophores increases the requirement for accurate spectral compensation of the data set becomes crucial.

Accurate size measurement allows for the possibility of using particle size as a means of encoding the beads. Previous work has shown that measurement of particle size is possible with an accuracy of ~50 nm using devices similar to those described in this work.³⁰ We are currently investigating the possibilities that an accurate size measurement allows, with respect to particle encoding technologies. The use of

microfabricated encoded microparticles for analysing multiplexed fluorescence assays on-chip is currently being investigated within our group.

Microfabricated devices offer a number of advantages over commercial FACS machines. The chips described here can be used as building blocks in the development of more sophisticated, flexible and configurable lab-on-a-chip devices. Such devices are capable of handling extremely small sample volumes and making measurements with high sensitivity, precision and accuracy.

Acknowledgements

This work was supported by Research Councils UK through the Basic Technology Programme (GR/S23513/01). The authors wish to thank Andrew Whitton for assistance with microfabrication, Shady Gawad and Raphael Tournay for assistance with the data acquisition software, and the clean-room staff at EPFL, Switzerland for fabrication

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