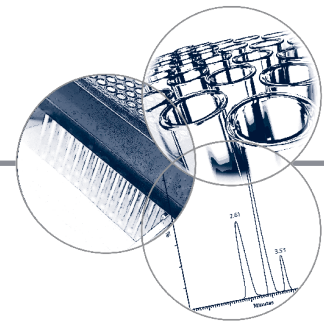

Recent developments in emerging microimmunoassays

Creative and novel microimmunoassay approaches continue to proliferate across many platforms originating from several fields of study. These efforts are aimed at improving one or more metrics for clinical tests, including improved sensitivity, increased speed, reduced cost, smaller sample size, the ability to analyze multiple antigens in parallel and ease of use. Many approaches focus on the production of microarrays that accomplish standard assays in parallel, or mobile solid-support formats to overcome issues of high background noise and long incubation times. In this article, innovative developments beyond existing commercial tests in the microimmunoassay arena are reviewed, covering January 2008 to April 2012. These developing experimental platforms are discussed in terms of their ability to augment or replace current commercial approaches.

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Over the last several years, microimmunoassay development has focused on replacing existing immunometric assays based on a microtiter plate format. Notable among these currently existing methods is ELISA. Since its introduction in 1971, ELISA has remained a core widely accepted practice and remains a common focal point to differentiate and discuss current experimental techniques [1]. Its success stems from the enzyme-based amplification mode and ease of use, along with the specificity and sensitivity of the antibody–antigen interactions common to all immunoassays. Despite the long incubation times, relatively high sample volumes and reagent costs, experimental assays aimed at the replacement of ELISA have universally failed to displace it, and few have been implemented commercially. While much of the following discussion is centered on comparisons with ELISA, all immunoassay platforms are included. Over the last 5 years, efforts to improve upon clinically used sandwich immunoassays have targeted one (or more) of six metrics; increased sensitivity [2–5], reduced analysis time [6–9], reduced cost [10], lower sample volumes [6,11], ability to **multiplex** [2,6,12–15] or operational simplicity [3]. While many studies improved various aspects of immunoassays, a so-called optimized immunoassay capable of displacing existing tests and significantly improving capabilities for clinical or diagnostic purposes has not been produced. However, several studies noted here may lay the foundation for a successor to ELISA.

Several groups continue to develop variations on standard immunoassay protocols, notably those of Ko, Gijs, Yang and Hage [16–22]. This current review will focus on the ability of these systems (and others) to create sensitive, robust, rapid and cost-effective diagnostic tools with high throughput on a multiplex format. While many of the assays discussed currently take place using singleplex analysis, their ability to be adapted to a high-throughput format will be assessed relative to other formats.

For any assay platform, the ultimate level of sensitivity will depend on the reaction kinetics (Keq) resulting from reagent quality [23]. However, reagent specificity will have differing impacts on the assay outcome depending on the platform in which they are used. An optimized clinical immunoassay format should meet several criteria to be applicable to a comprehensive range of diagnostic tests. First, a sensitivity down to the low pg/ml range is optimal so any plasma protein may be monitored, which allows for ultra-sensitive detection in medical diagnostics [24]. An analysis time should be no more than 1 h (if samples may be evaluated simultaneously) to permit changes over time to be tracked with ease [25], and sample volumes in the range of 10 µl per analyte interrogated to minimize reagent consumption. Finally, an assay should be able to multiplex for the evaluation of five proteins simultaneously, representing quantification of a group of biomarkers for a specific disease [15], and should take place with minimal transfer/pipetting steps to lessen variation between tests/testing sites [25]. These criteria represent the desired capabilities of an immunoassay platform such that it may be easily adapted for the detection of a complete range of targets in a clinical setting.

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Key Terms

Multiplex: Ability to simultaneously perform and quantify signals from several target compounds.

Fluorescent microsphere immunoassay: Technique that utilizes spectroscopically coded polystyrene spheres to perform the parallel analysis of up to 100 compounds from a single sample.

The individual assays discussed have been optimized for a particular target (or set of targets), which, in some cases, have requirements deviating from the desired qualities described above for an optimized clinical assay. While these criteria are not inclusive to all tests, they have been established to provide an organized framework in which to discuss the diverse experimental immunoassay platforms available.

While immunoassays have been the subject of several recent reviews [26,27], the focus of this review is immunoassay platforms aimed at improving diagnostic abilities published from January 2008 to April 2012, initiated with literature keyword searches associated with microimmunoassays along with the references and later citations of found works. These articles contributed new techniques to the field by improving LODs, decreasing sample analysis time, and refining the ability of assays to accommodate multiple samples in parallel. Many of the designs represent relatively simple fabrication processes and, while demonstrated for specific analytes, could be easily adapted for any number of target compounds. The topics addressed are categorized into three classes: use of micro- or nano-particles (NPs; both magnetic and nonmagnetic) as a solid support or to generate signal (section entitled 'Assays using microparticles or NPs'); generation of signal using flow conditions (section entitled 'Signal generation by flow conditions'); and, use of a static solid support to trap antigen and generate signal (section entitled 'Use of a static solid support to trap antigen & generate signal'). A summary of these techniques is provided for reference in Table 1. Although articles have been divided into these categories for clarity, they are not mutually exclusive and many studies could have been placed in more than one class.

Assays using microparticles or NPs

Assays employing microparticles or NPs may be further divided into two categories: those using magnetized particles and those that use nonmagnetic particles. Some of the techniques described here include the **fluorescent microsphere immunoassay** (FMIA) and its variations, magnetic bead-based immunoassays occurring fully on-chip where beads are manipulated to afford contact with successive reagents and samples, and magnetic bead-based assays that employ batch incubation off-chip prior to on-chip detection. The use of microparticles or NPs has gained popularity for a variety of reasons. Chief among these are the ease of manipulation during sample preparations and the ability to tailor the number of beads employed to suit the specific needs of an assay. This allows the solid surface area to be altered and optimized for various targets. Users are able to trap lesser sample concentrations on a small surface area (by employing low bead numbers), which provides signal concentration, allowing sensitive detection. Assays utilizing magnetic particles in particular also lend themselves well to coupling with varied signal processing approaches that have improved sensitivities and LLODs [4,5,28].

FMIA

FMIA is a technique that uses numerous sets of spectroscopically coded fluorescent microspheres, where each microsphere set is conjugated to a unique antibody or antigen, forming a solid phase for analyte detection [12]. This format was developed with a focus on multiplex analyses and has been used to successfully quantify ten compounds in parallel with detection limits in a clinically relevant range. Antigen–antibody reactions are simply performed in the well of a microtiter plate. Analysis follows, on a flow cytometer using Luminex® X-Map™ technology, in which separate wavelengths of light excite the microsphere sets and surface-bound reporter dyes [29,30]. The microspheres are labeled with a combination of red and orange fluorescent dyes. The ratio of these dyes acts as an identifier of the target analyte immobilized on the microsphere surface [25]. A separate detector, measuring green fluorescent response proportional to the amount of target, is able to quantify the total analyte present (Figure 1) [31]. This technique has been tailored to quantify groups of compounds relevant to a particular disease and possesses the obvious advantage observed in its ability to interrogate up to ten analytes from a single sample. However, in order to achieve clinically relevant levels of sensitivity, this format requires long incubation times and the use of specialized equipment. While verified assays are powerful in the information content they are able to provide, validation studies to ensure specificity represent a time-consuming hurdle for adaptations to limitless biological applications.

The labor-intensive development process for FMIA was demonstrated in one instance by the validation of assays detecting the porcine reproductive and respiratory virus in both serum and oral fluid-based samples [12,13]. This

work, conducted at South Dakota State University (SD, USA), produced an eight-plex FMIA for cytokine detection and requires a single 50 µl sample to quantify the analytes simultaneously [13]. Using the same volume typically required for one ELISA sample, the FMIA assay was able to achieve sensitivities in the pg/ml range. For seven out of the eight analytes studied, this detection limit represents between 1.2–8.2-fold improvement in sensitivity compared with the analogous ELISA. Follow-up work aimed at replacing serum samples with oral fluid resulted in decreased diagnostic capabilities [12]. However, the assay did maintain a clinically acceptable level of sensitivity and was able to detect the multiple target compounds in a single sample. While this work was successful in producing an assay based around the use of a noninvasive sample (allowing widespread testing), expansion to theoretical capabilities is limited by the quality, in terms of selectivity, of the antibodies employed. Additionally, since the possibility of shorter incubation times was not fully explored, leading to assay times comparable with a traditional commercial ELISA format [31], along with the requirement for sophisticated equipment, this assay carries significant costs both in terms of analysis time and labor for development.

Nonetheless, this technology has been used for various applications, including the detection of sera infectious agents [32], matrix metalloproteinases [33], and small-molecule drugs [34]. These uniquely optimized assays share the advantage of using a single sample for multiple analytes equivalent to the volume used for one

Table 1. Summary of the techniques of emerging microimmunoassays†.

Technique	Applications	Sensitivity	Analysis time	Equipment/fabrication requirements	Single-, poly- or multi-plex	Ref.
Non-magnetic particle assay						
FMIA	Protein/drug/ small-molecule quantification	pg–ng/ml	70 min–4 h	Luminex® beads; flow cytometer; Luminex X-Map™ technology	Multi	[12,13,27,30–37]
FIA	Protein quantification	~0.1 ng/ml	1–3 h	Fluorescent microscope; chip fabrication	Single/ poly	[14,50,52]
AlphaLISA®	Protein/toxin detection	~0.007 ng/ml	>1 h	EnVision® reader; AlphaScreen® beads	Single	[39,40]
BD Biosciences	Cytokine quantification	3 pg/ml	>3 h	BD FACSArray™ bioanalyzer; CBA kit	Multi	[38]
Off-chip incubation magnetic bead assay						
FIA	Protein/antibody quantification	~11.5 pg/ml	1–3 h	Microchip fabrication; fluorescent microscope	Single	[4,16,28,44,101]
Electrochemical detection	Antibody quantification	0.19 ng/ml	~3 h	Electrochemical sensor; microchip fabrication	Single	[45]
Chemiluminescent detection	Protein quantification	0.61 ng/ml	~2 h	Microchip fabrication	Single	[41]
On-chip magnetic bead assay						
Manipulation of particles	DNA, antibody, small-molecule quantification	~250 ng/ml–0.1 µg/ml	10 min–2.5 h	Fluorescence microscope; microchip fabrication	Single	[8,9,48]
Manipulation of reagents	Protein/ antibody quantification	3.2 fg/ml–16.4 ng/ml	35min–3 h	Microchip fabrication; electrochemical detector; isomagnetophoretic detector	Single/multi	[2,11,18,47]
Flow-based assay						
RDIA	Protein quantification	67 µg/ml	<10 min	Analog column; fluorescence detector	Single	[21,22]
Electrochemical detection	Protein quantification	1 pg/ml	40 min	Three-electrode electrical system; microchip fabrication	Single	[53]

†While this table provides an overview of technologies in the field, they represent average values for each category of assay, which give only a gross approximation for the capabilities of each immunoassay technique.
AlphaLISA: Amplified luminescent proximity homogeneous assay; CD: Compact disc; DRZ: Designated reaction zones; FIA: Fluorescence immunoassay; FMIA: Fluorescent microsphere immunoassay; NP: Nanoparticle; OLISA: Oligonucleotide-linked immunosorbent assay; RDIA: Reverse displacement immunoassay; SOFIA: Surround optical fiber immunoassay; SWIC: Series-wound immunosensing channels; TIRFM: Total internal reflection fluorescence microscope.

Table 1. Summary of the techniques of emerging microimmunoassays† (cont.).

Technique	Applications	Sensitivity	Analysis time	Equipment/fabrication requirements	Single-poly-multi-plex	Ref.
Static solid-support assay						
Capillary systems	Protein quantification	0.9 ng/ml^{-3} µg/ml	11–25 min	Microchip fabrication; fluorescence detector	Multi	[6,15]
Supercritical angle fluorescence	Cytokine quantification	4 pg/ml	13 min	Microchip fabrication; fluorescence detector	Single	[57]
SOFIA	Dye/protein quantification	~10 ag/ml	~3 h	Lock-in amplifier; optical fibers; photo-voltaic diode	Single	[5]
OLISA	Protein quantification	1 ng/ml	~3 h	Fluorescence detector; detection antibodies with differing fluorophore/quencher pairs	Multi	[56]
Portable disk automated ELISA	Protein/antibody quantification	0.51 ng/ml	30 min	Microdisc fabrication	Poly	[59]
Gyrolab™	Protein/antibody quantification	5 ng/ml	~1 h	Fluorescent detector; Gyrolab Bioaffy CDs	Single	[60]
DRZ chip	Protein quantification	5 ng/ml	~3 h	Microchip fabrication; fluorescence detector	Poly	[10]
NP-labeled array	Small-molecule/protein quantification	10 pg/ml	>3 h	Microchip fabrication; fluorescence detector	Multi	[55]
TIRFM system	Cytokine quantification	0.13 fg/ml	2 h	Microscope; microchip fabrication	Single	[24]
SWIC system	Protein quantification	0.6–0.89 ng/ml	~27 min	Chemiluminescence detector; optical shutter; microchip fabrication	Poly	[62]
Microcantelievers	Small-molecule quantification	0.1–1 ng/ml	>1 h	Photon sensitive detector; flow cell; beam splitter	Single	[26,27]

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ELISA target. Moreover, owing to reduced sample handling, the multiplexed estimates are less impacted by operator error as compared with ELISAs performed on multiple analytes requiring several trials [33]. These studies were able to achieve sensitivities of 1 µg/ml for sera infectious agents, 17 pg/ml for matrix metalloproteinases and below 1 ng/ml for small-molecule drugs. However, similar to the studies discussed above, antibody crossreactivity presents a practical limit to the number of analytes that may be interrogated from a single sample and may limit sensitivity.

Using a similar format, but with the added advantage of reduced analysis time, Kuriakose *et al.* described the use of FMIA for the development of a multiplex assay for avian influenza viruses [35]. The reactions were analyzed on a Bioplex instrument using a minimum of 100 microspheres in each set. Mean fluorescence intensity calculations for each bead set were used to quantify influenza viruses M, H5, H7, N1 and N2 to 0.04, 0.15, 0.17, 1.56, and 1.15 ng in a 50 µl sample, respectively. This represents an average detection limit of 12.3 ng/ml, where the assay can be accomplished within 70 min. While the detection capabilities of FMIA are not fully exploited in this effort, the study represents a subset of work where reduced analysis times hold greater importance.

A comparable emphasis on reduced analysis time is observed in the work on glycopolymer quantification by Pochechueva *et al.*, where analysis time totaled 90 min [36]. Here, glycoproteins Atri, Btri, Lex, and Hd were analyzed in both single- and multi-plex formats to assess antibody crossreactivity. The mono- and multiplex assay data correlated well, having Pearson's r-values ranging from 0.95 to 0.99 for the different analytes, indicating that the six target compounds investigated could be detected independently from a single sample. The lowest

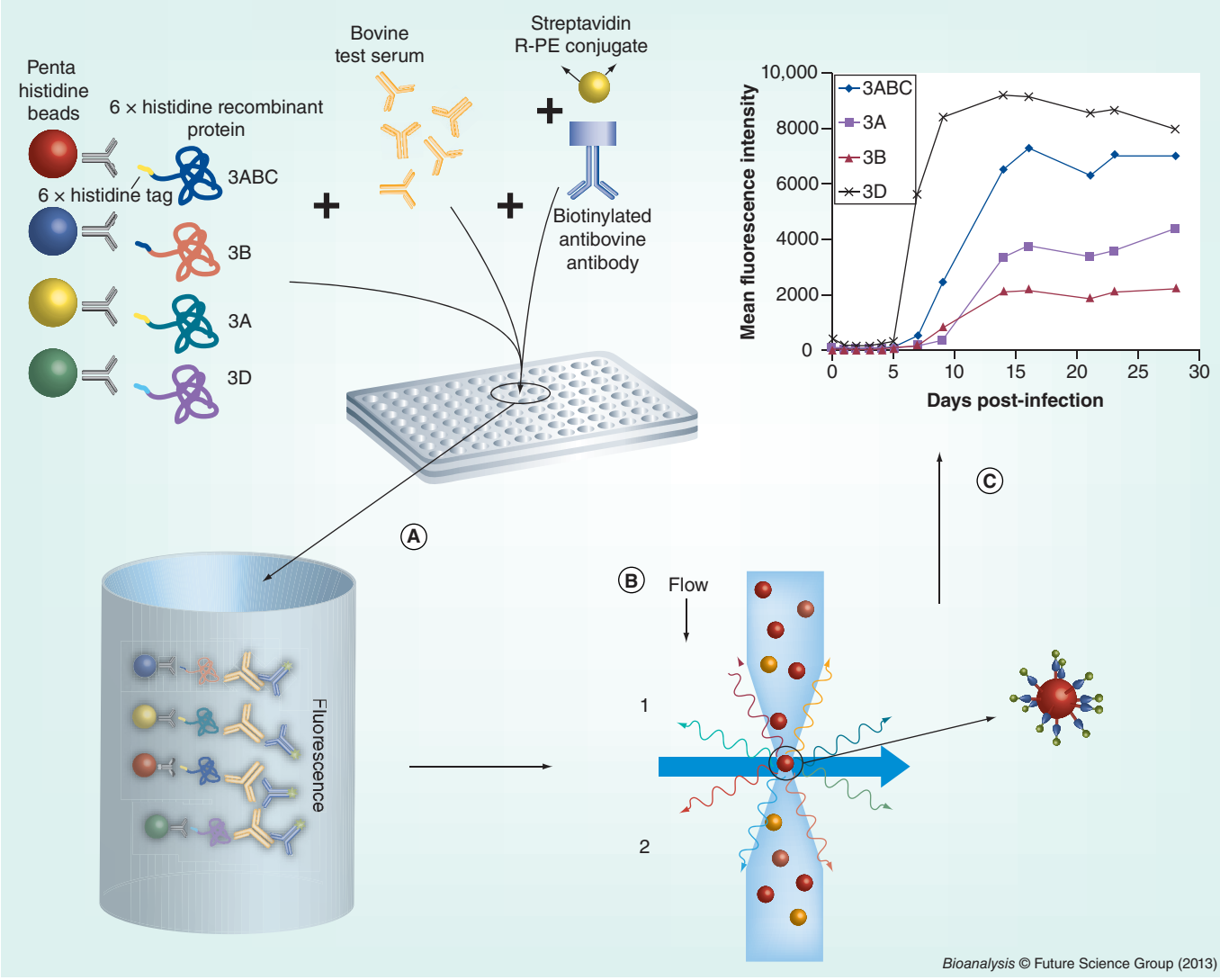

Figure 1. Overall process of a Luminex® multiplex immunoassay. (A) Following the immunoreaction, shown in fluorescence, signals from both the reporter-molecule and color-coding dyes are **(B)** read simultaneously and **(C)** processed digitally to translate signals to quantitative data. 1 and 2 indicate irradiation by two lasers using different wavelengths to excite both the fluorescent dye inside the microspheres and the detection antibody immobilized on the bead surface.

concentration tested was detected at 15 µg/ml. While successful under these circumstances, where target compounds have a relatively high relevant range, the full detection capabilities of FMIA were not maximized. Application to other biological targets, where physiological concentrations may be significantly lower, could require longer incubations and negate the time advantage observed here.

In a unique twist on work related to FMIA, Ji *et al.* reported on the production of quantum dot (QD)-doped microparticles for use in immunoassays [37]. A flow-focusing microchannel with a double T-junction was designed to merge a sodium alginate solution into a hydrogel matrix for trapping QDs. The system affords a series of QD-encoded microparticles to be developed in one step. When tested in an immunoassay on IgG, fluorescein isothiocyanate-labeled IgG could be detected to a minimum concentration of 2.2 µg/ml. Further investigations are needed to separate target and encoding signals and optimize other assay conditions to improve detection sensitivities. Once optimized, this process provides an attractive alternative to the need to purchase fluorescent microparticles commercially for small-scale operations focused on minimizing cost. However, the low cap on bead diversity, as well as the time required to produce the QDs, limit the utility of this platform for large-scale clinical use.

The assays developed using FMIA have, to this point, achieved success in measuring up to ten analytes from a single sample [32]. They require low sample volumes (typically 50 µl), which has permitted the thorough investigation of limited samples by allowing quantification of anywhere from one to ten antigens. Each of the investigations discussed have successfully adapted FMIA to suit their individual needs. However, they are relatively expensive to perform due to the requirement for specialized analysis equipment and depend completely on antibody specificity for a reliable response. This reliance on antibody quality restricts the flexibility of FMIA in terms of adaptation to new target compounds since adjustments require extensive testing to assure minimal crossreactivity within the assay. This immunoassay format also requires long incubation times, totaling 2 or more hours, in order to achieve levels of sensitivity in the pg/ml range. The requirement for these long incubations arises from sample preparation. Samples are incubated in the absence of convective mixing or sample flow employed by other methods to increase the speed of antibody–antigen recognition events.

Other companies have developed similar commercial products to FMIA, including the cytometric bead array from BD Biosciences (NJ, USA) and the amplified luminescent proximity homogeneous assay (AlphaLISA®) [38–40]. Like FMIA, cytometric bead array uses small sample volumes (50 µl) and has achieved clinically acceptable LODs (3 pg/ml) while operating in the same time frame as FMIA [38]. AlphaLISA has been able to quantify target compounds in a shorter time span using a competitive assay format [39,40]. While AlphaLISA has also attained sensitive detection (0.007 ng/ml) in a shorter time frame using a competitive assay format, signal production depends on an energy transfer between donor and acceptor beads in close proximity to produce a chemiluminescent signal, which subsequently activates a fluorophore in the same bead. This method of signal production has resulted in studies that focus on assay development in a singleplex format. Adaptation to a multiplex format would require the ability to distinguish between signals from different acceptor beads. Additionally, because AlphaLISA operates in a competitive assay format, where increases in analyte represent decreases in observed signal, the LODs for this platform are not as sensitive as those operating in a noncompetitive sandwich assay design.

■ Off-chip preparation of magnetic bead-based assay

The use of magnetic particles as a solid support is an attractive alternative to fluorescent microspheres because it allows for easy manipulations and separations both on- and off-chip. Off-chip incubation is often employed because it allows sample preparations to be performed in advance of the assay. The initial incubations are simple to perform and can be accomplished using common laboratory equipment, such as an Eppendorf tube [28] or the well of a microtiter plate [41]. Magnetic particles may be easily detained by the introduction of a permanent magnet during wash steps, and high sensitivities have been achieved with small sample volumes. In addition to the ease of manipulations and high sensitivity, use of magnetic particles has gained in popularity because of their compatibility with diverse detection and signal processing systems, including, but not limited to: chemiluminescent [41,42], fluorescent [16,17, 28 ,43] or electrochemical [44] detection.

Fluorescence continues to be one of the most popular detection methods, and several protocols used in fluorescence immunoassays (FIAs) were described during this time period by the Gijs group from Switzerland [16,17]. In one article, a channel was constructed having periodically enlarged cross-sections used to trap magnetic chains in a homogeneous field [16]. The results showed that off-chip incubation of capture antibody with target analyte under agitation produced uniform fluorescence throughout the channel (Figure 2). This approach provided a LOD of 50 ng/ml, which is similar to classical ELISA. However, the off-chip incubation resulted in the linking of beads via capture antibody interactions creating chain irregularities on-chip. By implementing a full on-chip procedure, the issue of chain irregularities was resolved [16,18]. This gave an improved LOD of a few ng/ml and afforded a reduction in assay time from 2 h to 25 min.

Building from their work on FIA protocols, an integrated silicon chip was developed by Dupont *et al.* based on the measurement of photon-induced electrical current pulses in single-photon avalanche diodes [17]. This allows for fluorescence measurements of microparticles without the requirement of a microscope. Here, manipulation on-chip after off-chip incubation of sample is achieved by applying current through microcoils, which positions single beads over a single photon avalanche diode.

Once oriented, the fluorescence signal of a single bead could be measured for the detection of monoclonal antibodies down to 1 ng/ml in only 25 min using a sample volume of 100 µl. While this assay achieves a comparable sensitivity in the same time frame to the full on-chip assay described by the group, the sample volume required is much greater (100 µl compared with 4.1 nl) [16,18]. Additionally, the speed of this assay is an improvement over the 2.5–3 h typically required of a commercial ELISA using an identical sample volume. However, the LOD is slightly higher than the 0.03 ng/ml limit typically observed commercially for monoclonal antibodies used in analyte capture [101].

A different approach to signal generation in FIA protocols is described by the Hayes group from Arizona State University [4,28]. In these articles, during data acquisition on an inverted fluorescent microscope coupled to a CCD camera, a magnetic field is introduced. By incorporating a periodicity into this field, lock-in amplification was used to selectively quantify surface-localized myoglobin, even in the presence of background noise. Using lock-in amplification, a LOD of 1 ng/ml was afforded, which is comparable to the methods previously described [28]. By introducing a novel image processing system capable of estimating and eliminating background noise, only the pixels corresponding to the solid surface are used in concentration determinations [4]. Coupling this signal processing to the previously described immunoassay protocol improved sensitivity to a 11.5 pg/ml detection limit for myoglobin using a sample volume of 30 µl. This detection limit represents roughly 100-fold improvement over previous results and is 2.3-fold more sensitive than the corresponding ELISA.

Off-chip incubation protocols have also been described for methods using varied detection methods. Electrochemical detection was employed both by Proczek *et al.* [44], and by Piao *et al.* [45]. In the work by Proczek *et al.*, analyte quantification was performed using GRAVITM-chips from DiagnoSwiss [44]. These chips contain eight independent microchannels, which allows parallel testing. Following off-chip incubation, IgE could be quantified to a detection limit of 17.0 ng/ml in less than 1 h.

Piao *et al.* used a novel approach to develop an electrochemical immunosensor based on carbon nanotubes coated with enzyme and magnetic particles in combination with an electrically driven reversible reaction allowing substrate recycling to amplify the signal [45]. After off-chip conjugation of magnetic particles and capture antibody to the carbon nanotubes and the binding of target analyte on-chip, the sensing assembly is magnetically guided to a gold electrode. Here, the amperometric responses of the enzymatic reaction were recorded using cyclic voltammetry. Results show a LOD of 0.19 ng/ml of hIgG after a 30 min enzymatic reaction. While both methods were able to quantify target compounds with a similar LOD to FIA that do not require the use of a fluorescent microscope, sensitivity is afforded through long enzymatic reactions relative to assays boasting the completion of entire protocols within 25 min [16,18].

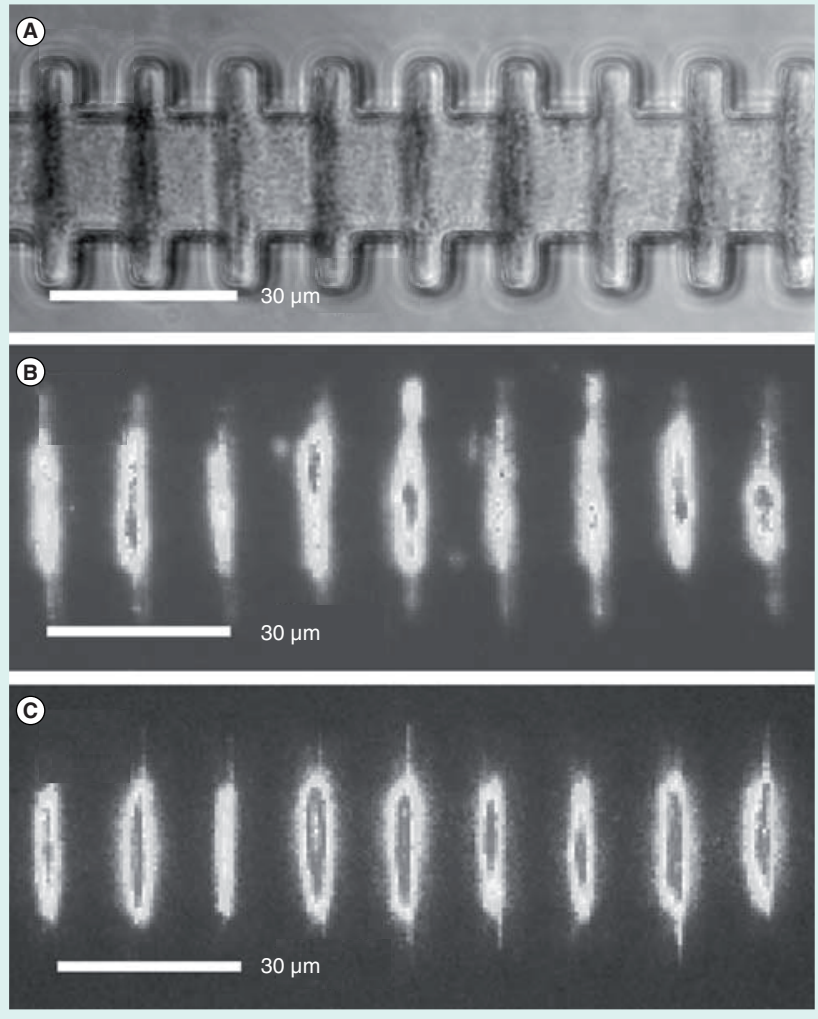

Figure 2. Images of the self-assembled chains formed using off-chip incubation, on-chip detection and the full on-chip immunoassay formats. **(A)** Shows an optical image of the self-assembled chains following off-chip incubation. **(B & C)** Compare the fluorescence images of the chains after the off-chip incubation and full on-chip assay, respectively.

An alternative method employing batch incubation was described by Li *et al.* [41]. Here, the development of a microplate magnetic chemiluminescence immunoassay was discussed. This assay uses magnetic particles as the solid support and microplate wells as the reactor. The full procedure takes just under 2 h to perform. With incubation times similar to conventional ELISA, a LOD of 0.61 ng/ml for carcinoembryonic antigen was afforded using a sample volume of 35 µl. Although not offering advantages in terms of rapid analysis, and with a format that would be reliant on antibody quality to preserve sensitivity if multiplexing were to take place, this assay affords a competitive detection limit while requiring roughly a third of the sample used by current commercial protocols.

This group of assay methods shares the advantage of sensitive detection limits using affordable methods and, in general, low-to-moderate levels of complexity. They also offer the ability to limit sample use (generally between 30–50 µl is consumed) and reagent consumption. This is afforded by the ability of the magnetic particles comprising the solid support to remain free-flowing during incubations, as well as through convective mixing, which allows the entire sample to be interrogated for antigen capture affording quantifiable signal of low-concentration targets from small sample volumes. Additionally, while many have been evaluated only in a singleplex format, the alteration to these assays allowing the ability to multiplex is straightforward and should not affect assay quality. However, while many of these assays require only simple laboratory equipment for the initial incubation steps, the chips employed during detection (as well as the detection methods themselves) vary greatly. Therefore, while many methods can be performed with the use of a common fluorescent microscope, there may be initial instrumentation costs depending on the assay platform selected. Furthermore, with off-chip preparation of samples, long incubation times in the order of hours are required. This limits the capability of these assays to make serial measurements and track concentration fluctuations with time. In addition, some studies have observed issues in the manipulation or non-uniform aggregation of beads on-chip, following off-chip pelleting protocols during wash steps [16]. Many of these issues can be eliminated through the adaptation of batch incubation procedures to those that take place fully on-chip. The advantages, and limitations, of the on-chip immunoassay format are discussed in the following section.

■ On-chip assay with sequential introduction to reagents/samples

When immunoassays take place entirely on-chip, the magnetic beads employed as the solid support may be manipulated in a variety of ways. Beads can be injected onto the chip at the outset of the experiment, immobilized by permanent magnets, and introduced to reagents and sample by sequential injection [11,16,18,46,47]. They may also be injected onto the chip and manipulated through static plugs of sequential reagents [48], or forced through laminar streams of flowing reagents [8,9]. Relative to their batch-incubation counterparts, these assays are relatively simple to perform, requiring minimal pipetting steps and no transfer of the assay between containers. This minimizes the aggregation issues that have been observed in some off-chip immunoassay applications [16]. Additionally, through the flow of sample and reagents, the duration of the assays is minimized. While this is sometimes accompanied by a decrease in sensitivity, some optimized procedures are able to remain competitive with those using longer incubation steps.

The manipulation of magnetic particles through streams or static plugs of sample and reagent was explored by several groups [8,9,48]. In the first study, performed by Sasso *et al.*, magnets are placed on both sides of a microchannel [9]. The field is strong enough to pull magnetic beads to the wall of the channel, but not strong enough to overcome the shear stress from fluid flow required to trap the particles. This allows incubations to occur along the channel walls, and the beads are able to traverse the channel to enter or exit reactant streams. This format allows rapid assay times and requires minimal handling of the sample or reagent. Using an epifluorescence microscopy detection platform, a 625 ng/ml LOD was realized for biotin–fluorescein isothiocyanate with incubation times of less than 5 min and a sample volume of 90 µl. Despite requiring a relatively larger sample volume compared with other magnetic particle-based assays, this study allows for rapid serial measurements. This could easily be used to track changes in analyte concentration with time, but only for target compounds with a high concentration in plasma. Alterations to the method would have to be made to afford more sensitive detection, and allow this method to be readily ported to additional applications.

A second example of the rapid analysis afforded by fully on-chip immunoassay applications was described by Peyman *et al.,* where IgG quantification was achieved in about 10 min consuming only 7.5 µl of reagents [8]. In this study, several independent laminar flow streams are produced across a rectangular reaction chamber. The functionalized magnetic particles are deflected across these streams, passing through sample, wash and detection reagents. Once the chip is set up there is only one required pipetting step to perform the assay, minimizing variations between runs. This, in addition to speed, represents a secondary advantage over batch incubation processes. Results show that negative controls used on-chip produce little to no nonspecific binding or transfer of reagents between boundaries, evidenced by the lack of fluorescence for these samples. Using this system the LOD for IgG was 0.1 µg/ml. This high LOD could potentially be improved by increasing the sample volume, or through longer interaction times of the magnetic particles with the sample.

A final example of particle manipulation through reagents and sample was developed by Chen *et al.* from the University of Rhode Island (RI, USA) who describe a platform for a microfluidic inverse phase ELISA [48]. In this format, magnetic beads are loaded into a microchannel and transferred sequentially through plugs of sample and reagents separated by oil. This design allows the assay to be set up completely ahead of time and allows the process to be limited to one pipetting step, making the operation simple. The oil plugs also prevent the mixing of reagents before and during the assay. The beads are allowed to incubate in each plug for 30–45 min and fluorescence data are collected for 180 s after being moved into the final buffer plug containing a fluorescein diphosphate solution. Using this platform, digoxigenin-labeled dsDNA was detected to a limit of 259 ng/ml. However, at higher sample concentrations, the microfluidic inverse phase ELISA was less capable of detecting analyte compared with traditional methods. This was proposed to be a product of carry-on water between plugs bringing free detection antibody into the exposure plug. Although slightly more sensitive than similar on-chip methods, this assay loses the advantage of rapid analysis and does not compare to the sensitivities achieved with similar incubation times off-chip. Additionally, adaptation of the current assay to a multiplex format would involve use of all four parallel channels available in the current chip design. This would allow analytes to be quantified simultaneously, but would quadruple the consumption of sample and reagents compared with the current system.

The other predominant assay structure for on-chip protocols involves maintaining magnetic beads in a single position by employing a homogeneous magnetic field and sequentially introducing reactants by flow, which has been extensively explored [2,11,18,47]. Keeping particles trapped in a magnetic field prevents undesirable aggregation and reduces the loss associated with particle transfers using batch incubation. In addition, sample and reagent exposure times can be varied simply by altering flow rates to optimize signal under minimally required assay durations. This minimizes assay times while affording LODs competitive with assay formats requiring long incubations. This approach was used by Do *et al.* from the University of Cincinnati (OH, USA) to design a new lab-on-a-chip facilitating an enzyme-labeled electrochemical immunoassay [11]. The chip uses a magnetic microarray as a bead separator and an interdigitated array microelectrode as a biosensor. Results show IgG could be detected to 16.4 ng/ml in 35 min using 5 µl of reagent.

In another study, magnetic NPs were used as labels on microbeads to detect bound analyte by isomagnetophoretic focusing [2]. An external magnetic field causes particle movement to a denser or sparser field until its magnetic susceptibility is equal to the surrounding gradient. This is important because it allows small changes in concentration to be detected by utilizing a low concentration of gadolinium paramagnetic diethylenetriamine pentacetic acid (Gd-DTPA), used to create the magnetic susceptibility gradient. This low concentration allows a narrow dynamic range with high resolution. However, by employing a higher concentration of Gd-DTPA solution, a wider concentration range may be interrogated, making the assay flexible for diverse target compounds. Using this set-up, rabbit IgG-biotin could be detected to a limit of 3.2 fg/ml. The use of fluorescent microbeads allowed for a multiplexed assay with the detection of three analytes while maintaining pg/ml sensitivity and requiring 200 µl of sample. This represents improved sensitivity compared with commercially available ELISA, but requires double the sample volume and a similar assay duration. With the flexibility of tailoring the

Gd-DTPA gradient, and using long incubations during sample preparation and specialized equipment to prepare and analyze samples, a LLOD was achieved for this assay.

Many of the fully on-chip immunoassays afford users rapid results and consume low volumes of sample [8,9,11]. Rapid analysis is allowed by manipulating the solid phase through reagents or by holding the solid surface in place while flow is used to direct sample to the assay surface, decreasing the depletion zone observed with diffusion-mediated incubations. Large depletion zones that reach a sensor-size dependent steady state during incubations dependent upon diffusion can be combated by convective mixing or flow, which accelerate mass transport and actively decrease the thickness of the depletion zone near a sensor surface [49]. This allows the assay time to be dependent upon the speed of the reaction itself as opposed to mass transport limitations. However, this rapid analysis is frequently accompanied by higher LODs. While nonspecific binding is not a large problem because the particles are in contact with the sample and reagent for short time periods, the entire population of antigen may not be trapped, causing an increase in LODs. In other cases, long incubation times have allowed sample analysis with high sensitivity and the interrogation of multiple analytes [2]. Where optimal incubation times are employed these assays require less sample manipulation than their corresponding off-chip counterparts and have shown equal, or greater, sensitivities. Similar to their off-chip counterparts, many of these assays may be accomplished using a fluorescent microscope as the detection element. Nonetheless, due to the diversity of assay platforms, many formats require specialized equipment to perform. This, along with the need to fabricate chips on a large scale, could increase the initial cost and time investment in adaptations of the techniques to a large scale. The full on-chip assay structure holds promise both in terms of assay sensitivity and rapid analysis. However, in order to produce a truly optimized assay, these considerations must be balanced to afford a test capable of interrogating any biological sample of interest, regardless of the targets' physiological concentration.

■ Other techniques

Several studies have employed microparticles or NPs in creative ways that do not fit into one of the above categories. These include rapid analyses where particles are spiked directly into a sample for target quantification [3], protein-functionalized microparticles capable of electrostatic self-assembly [19,20], and fluorescent microbeads that employ simple detection methods [50]. These varied techniques hold individual advantages specific to their applications. Some have been tailored for the rapid analysis of target compounds, while others have been simplified to allow ease of use. The pre-eminent disadvantage associated with the assays described below is their vast differences from other microbead assays, requiring large adaptations in the average laboratory for widespread implementation.

In the study by Ranzoni *et al.*, a new technology based on magnetic NPs in a pulsed magnetic field was investigated [3]. This method uses a small spike of NP-probing reagent, precoated with monoclonal antibodies, which is directly injected into a sample. The particles are free to move within the sample to capture antigen without the presence of a magnetic field. By introducing a pulsed magnetic field, the particles are concentrated and allowed to form clusters mediated by biomarker-induced interparticle binding. These clusters are then detected by applying magnetic rotation frequencies and using optical scattering to determine cluster size, which correlates with antigen concentration.

Using this technique, after only one reagent addition step, an assay can be performed in a total time of 14 min. Using this scheme, prostate specific antigen was detected to a limit of 13.6–17 pg/ml in plasma. This format allows for the sensitive and rapid quantification of a single analyte, in a format greatly simplified compared with ELISA testing. The analysis of multiple compounds would depend on the specificity of antibodies, comparable to FMIA. It would also require detection to be altered so that clusters possessing different targets may be identified without the addition of sophisticated analysis equipment.

Another study based on the manipulations of magnetic microparticles was described by Afshar *et al.* in the development of a microfluidic magnetic actuation system that allows the 3D focusing of magnetic beads for agglutination assays [51]. The system was designed with a magnetic microtip, used as a field concentrator, to focus magnetic beads in a microchannel. A single lateral sheath flow positions and aligns individual beads in the center of the flow. This allows a small number of beads to be counted in an observation window by automated image

reading. Having the individual beads 3D focused in the flow center allows reliable counting of single beads versus agglutinated bead doublets, which allows biotinylated bovine serum albumin concentrations to be determined. It was demonstrated that biotinylated bovine serum albumin could be detected to 400 pg/ml (6 pM) with the fully on-chip assay in about 20 min with the consumption of a 2-µl sample. This format, while rapid, would be difficult to multiplex due to the quantification of signal arising from the counting of aggregate numbers.

As an alternative to the use of magnetic beads, the Gijs group contributed several immunoassay articles investigating electrostatically self-assembled micropatterns performed on-chip [19,20]. Electrostatic forces were used to mediate bead self-assembly in a channel formed by reversibly sealing polydimethylsiloxane (PDMS) onto an (aminopropyl)triethoxysilane patterned glass substrate. As opposed to external magnets that create dense bead plugs on-chip, the fabrication of positively charged (aminopropyl) triethoxysilane-patterns results in low fields where beads align. This allows the formation of self-assembled chains that are stable during both flow-based and static incubation steps. Performing the immunoassay in stop-flow mode, where the channel is sequentially filled and incubated with sample and reagents, afforded IgG quantification to a lower limit of 15 ng/ml in 30 min using 560 nl of sample.

A second contribution investigated the effect of continuous-flow versus stop-flow conditions for the assay [19]. The results show that mouse antigen (m-Ag) could be detected under continuous flow to a limit of 250 pg/ml, representing roughly a 60-fold improvement over stop-flow limits and requiring only 10 min to perform. This procedure was performed using 1.3 µl. The advantage of reduced analysis time is afforded using continuous flow because diffusion-associated depletion of analyte around the bead chains does not occur as observed under stop-flow conditions. This allows more analyte to be successfully captured onto beads in a short time span, analogous to analyte capture in affinity chromatography utilized during protein purification. Additionally, high specificity antibodies allowed for detection of two analytes on a single chip. In both cases, the rapid analysis and small sample size provide advantages compared with ELISA without sacrificing the LOD. By decreasing the flow rate, thereby increasing analysis time, the second assay could potentially reach a more sensitive LOD. This would maintain its advantage of small sample requirements and rapid analysis while increasing its ability to compete with more sensitive analysis techniques.

Several studies employed simple polystyrene spheres in unique ways to produce fluorescent signals. In the study by Fu *et al.*, a bead-trapping/releasing flow cell for a fluidic assay was developed [52]. This device integrated a pillar-array and pneumatic valve to provide flow injection/sequential injection analysis. Using the valve, beads could be manipulated in the device to perform the immunoassay in 10 min with a detection limit of 0.80 ng/ml for 3,4,6-trichloropyridinol using a competitive assay format and 15 µl of sample. This assay could later be altered to perform a noncompetitive assay, which would improve the sensitivity but increase the time required for analysis. Even with these alterations, the current detection method does not lend itself easily to multiplexing and would have to be altered to distinguish between signals arising from different compounds in order to quantify multiple antigens in parallel.

A second example of polystyrene microsphere use is the crosstalk-free duplex FIA for the simultaneous detection of carcinoembryonic antigen (CEA) and neuron-specific enolase described by Cao *et al.* [14]. A sandwich immunoassay was developed using multiple QDs as detection elements, which yield a tunable, symmetrical and narrow emission band. Each color QD conjugate was capped by a capture antibody, and polystyrene microspheres brought antibodies proximal to the QD surface in a diffusion-driven incubation, allowing both antigens to be specifically identified simultaneously to a limit of 0.625 ng/ml, which is comparable to many methods using long incubation durations. The assay requires incubation times analogous to ELISA, but could be further multiplexed depending on the specificity of antibodies employed.

Finally, employing intrinsically fluorescent beads in a unique way, a fluoro-microbead guiding chip-based sandwich immunoassay for the quantification of biomarkers was investigated by Song *et al.* [50]. The fluoro-microbead guiding chip consists of four immunoassay regions, each containing five gold functional surfaces to support five identical tests performed simultaneously with the quantification of four separate analytes in parallel. The gold surfaces were conjugated to capture antibodies to create a sensing surface, where capture of both antigen and detection antibody was diffusion-driven.

Key Term

Reverse displacement immunoassay: Rapid technique that quantifies a single analyte with a turnaround time of minutes.

Using fluoro-microbeads conjugated to an antibody and a fluorescent microscope, a sandwich immunoassay was performed and antigen concentrations were determined directly by counting microbeads immobilized on the immunosensing regions. In an assay time of less than 1 h, cardiac troponin I could be quantified in a range from 0.1 to 100 ng/ml. With the addition of agitation or convective mixing to increase sample capture and improve sensitivity, its time advantage over ELISA may be maintained.

As illustrated in the sections above, the immunoassay applications achieved using both magnetic and nonmagnetic particles as a solid support are diverse. These assays have been tailored to meet the needs particular to their use, which may be increased assay speed, sensitivity or simplicity of operation. In general, assays that achieved rapid analysis suffered from limitations in sensitivity. However, assays have not been produced that optimize the relationship between assay speed and ability to detect biomolecules with diverse physiological concentrations. Research to improve this relationship may be the most promising avenue toward reaching a truly optimized microimmunoassay, owing to the other advantages inherent to microparticle solid supports, such as ease of manipulation, small sample size, and the straightforward coupling to advanced signal processing methods.

Signal generation by flow conditions

A second major area of interest in microimmunoassay applications involves those based around the use of flow conditions to produce a quantifiable signal. In this section, assays employing microcolumns [21,22] and immobilization on channel walls [53], where target quantification is achieved by the release and flow of a signal-generating agent to a detector, will be discussed. This format has the advantage of allowing rapid quantification times with small sample requirements. Additionally, it is easily adapted to quantify different analytes. However, these assays are limited to a singleplex format and each antigen would require a unique column.

The Hage group from the University of Nebraska has contributed significantly to this area since 2008 [21,22]. One recent study introduced a **reverse displacement immunoassay** that generates signal by the analyte displacement of a label from a small immobilized analog column [22]. When a complex is formed between analyte and label, a displacement peak is created and the signal is measured allowing analyte quantification (Figure 3). Results show the LLOD for reverse displacement immunoassay to be around 67 µg/ml (27–29 pmol) and the ULOD 400 µg/ml (160–200 pmol) for a 20 µl sample of mouse IgG Fab1. The total assay analysis time is less than 10 min, with signal generation occurring within 20–30 s after sample application to the column. This rapid analysis, which offers a pronounced time advantage over ELISA, is afforded because no pre-incubation of the sample with label is required. While this assay is limited to a singleplex format, it can be applied to any analyte where an appropriate label and immobilized analog are available or can be generated. Although sensitivity of the assay may be improved by using a larger sample volume, this format is not competitive with those employing incubation steps between sample and detecting agent.

A second contribution made by this group analyzed the binding and elution of target compounds from immunoaffinity chromatography/high-performance immunoaffinity chromatography columns in order to understand both association and dissociation efficiencies [21]. Using this format, a variety of detection schemes can be used to obtain kinetic and binding information, including fluorescence, MS and absorbance. The insight gained from this study can be valuable in the design of future solid-phase immunoassays.

A more sensitive flow-based assay was described by Liu *et al.* who published on the development of a poly(methylmethacrylate) microfluidic chip coupled to electrochemical detection for the quantification of α-fetoprotein (AFP) [53]. AFP antibody is immobilized on the poly(ethyleneimine)-derived poly(methylmethacrylate) surface. After antigen and horseradish peroxidase-conjugated AFP antibody bind sequentially in the channel, a three electrode electrical system at the microchip outlet records the reduction in the H_2O_2 current response. Results show a linear response between 1 and 500 pg/ml with a 1 pg/ml detection limit requiring minimal use of sample in a time of 40 min. Although it requires minimal sample and achieves sensitive quantification in under 1 h, the assay is not easily adaptable to the analysis of multiple analytes in parallel.

Theoretical work to assist in predictions of device performance was conducted by Sinha *et al.* [54]. A comprehensive model was created to characterize interactions during a flow-through immunoassay. Findings may help provide a rational basis for determining operating conditions

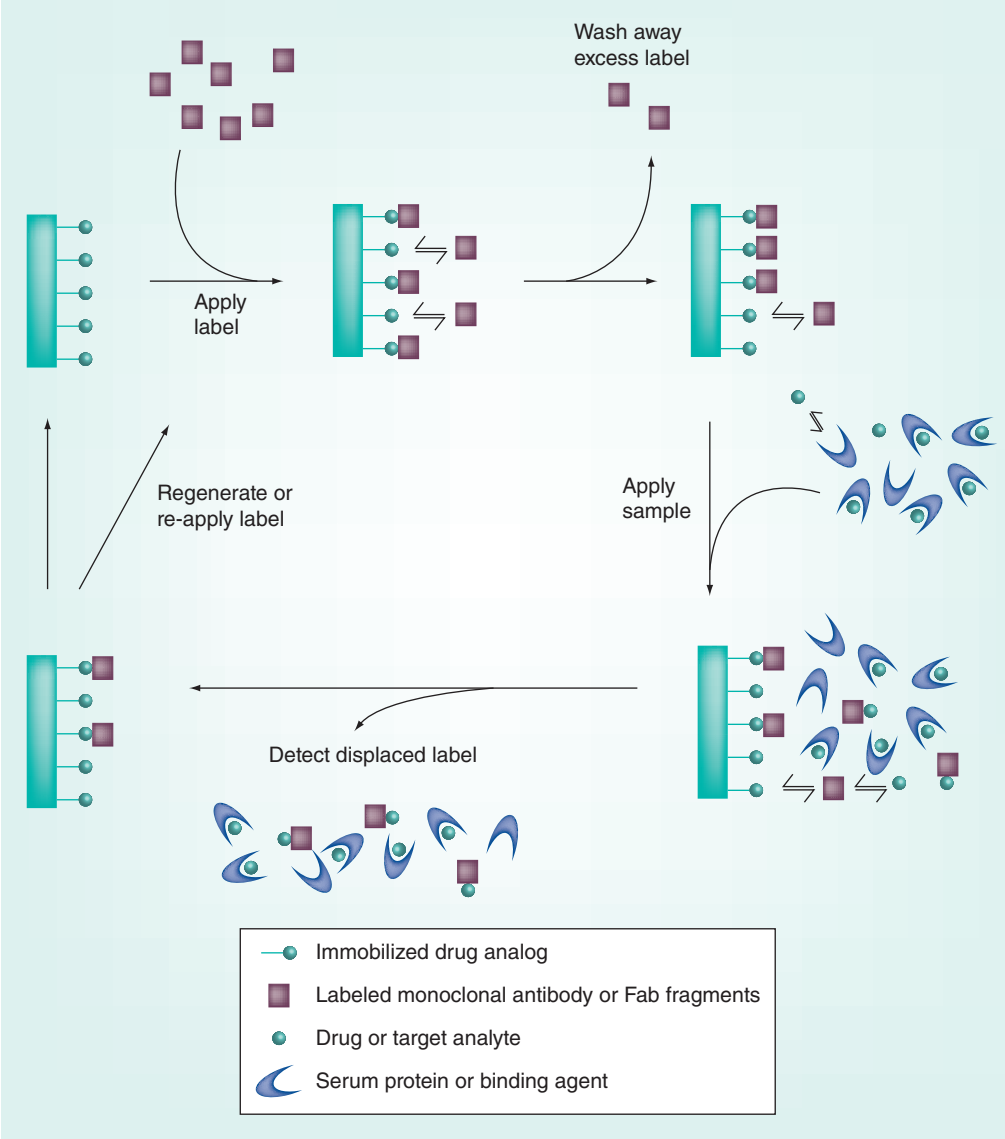

Figure 3. Scheme for a reverse displacement immunoassay.
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in microfluidic ion-mobility spectrometry devices.

These immunoassays share the ability to achieve the rapid quantification of analytes using minimal sample volumes. This rapid analysis is made possible by the ability to perform the assay without sample pre-incubation steps. The sensitivity of these rapid tests is comparable to many immunoassays employing much longer incubation times [14,45,48], and are easily adaptable to quantify any target compound. However, since detection is dependent on the measurement of a displacement peak composed of label and antigen, multiplexing this assay format would be challenging.

Use of a static solid support to trap antigen & generate signal

The use of a solid support provides certain advantages, including the straightforward ability to multiplex and sensitive LODs. In this area of research, many variations on this traditional 'static well' format have evolved. This section discusses techniques that employ antibodies patterned on PDMS [6,7] or in capillary systems (CSs) [15], as well as antibody microarrays [55], and novel techniques that employ static detection, such as the surround optical fiber immunoassay [5] or oligonucleotide-linked immunosorbent assay (OLISA) [56]. These assays offer the advantage of easy and direct multiplexing,

often accomplished by the patterning of capture antibodies on a static surface. Many of the assays also offer sensitive LODs owing to long incubation times. The long incubations allow time for sample to interact with antibodies by diffusion, but put a limitation on the potential throughput or ability to track changes in protein levels over time using serial measurements.

Since 2008 many groups have developed new technologies centered around the traditional static solid-phase immunoassay. The Delamarche group from Switzerland has made multiple contributions in this area [6,15]. In one study, the patterning of capture antibodies (cAbs) on PDMS in order to be compatible with CSs was described [6]. Once cAbs are patterned, the PDMS block is placed on CS with cAbs oriented perpendicularly to the reaction chambers, which produces well-defined areas for analyte capture from solution. These small patterned areas allow one-step fluorescent imaging of all analytes, and the fast reaction is permitted by confining sample to a minute space as it flows over capture zones. The capillaries allow multiple analytes to be detected from small samples (1 µl or less). This produces a 'micro-mosaic assay' with the potential for 96 test sites if used with a chip having six independent reaction chambers. Results show that CRP could be detected to a sensitivity of 0.9 ng/ml in 11 min using only 1 µl of sample. While already comparable in sensitivity to ELISA with a much smaller sample and shorter assay duration, the sensitivity of these assays may be further improved by coupling the detection to signal-amplification methods.

In a second contribution, the group described a one-step immunoassay using CS [15]. The assay is based on the preloading of freeze-dried detection antibodies into the analyte flow path. After antibody reconstitution and analyte addition, fluorescence detection can be performed downstream on patterned capture antibodies. Results show that within 10 min, analyte concentrations with a lower limit of 3 µg/ml could be detected. After 25 min of total assay time a decrease in the background noise (resulting from the decay of unbound detection antibodies) allowed concentrations down to 1 µg/ml to be observed. This single-step assay reduces handling overhead for the end user. Although this assay possesses a higher LOD and an equal or longer assay time than the previous work, this study suggests that the positive aspects of CS previously exploited could potentially be achieved in a one-step immunoassay.

The fluorescent one-step immunoassay platform was further studied by Ruckstuhl *et al.* [57]. In this contribution, a system of polymer test tubes with fluorescence collection optics was utilized along with a compact fluorescence reader. The detection technology, based on supercritical angle fluorescence, allows for the real-time monitoring of surface reactions. The intensity of the signal decays exponentially with the distance from the boundary; therefore, surface-selective detection is achieved providing a sensitive readout for immunoassays. In an assay time of only 13 min, IL-2 could be quantified with a linear response down to 4.5 pg/ml using a sample volume of 40 µl. This represents advantages compared with the traditional ELISA format, which requires 4 h and 100 µl to give a LOD of 4 pg/ml, but presents an analogous limitation in terms of multiplexing. The assay, currently taking place in disposable test tubes, may be adapted to a well-plate format, but will remain limited to the analysis of one compound per sample.

Other static solid-phase assays produced based on the traditional format continue to employ 96-well microtiter plates as incubation chambers. Chang *et al.* reported on the development of surround optical fiber immunoassay, which uses 96-well plates for incubations, consuming volumes analogous to commercial ELISA methods [5]. Analysis takes place in a singleplex format using specially designed equipment. Samples are placed in a 100 µl microcapillary and excited in a detection unit by focusing temporally modulated light along the capillary's axis. After light is focused into a single optical fiber and coupled to a low noise photo-voltaic diode, detection takes place using phase-sensitive detection employing a lock-in amplifier. The sensitivity was tested using rhodamine red and results show a 0.1 attogram LOD. Prion proteins from varying species were also investigated and found to have a LOD >10 attograms from a sample volume of 100 µl and requiring long incubation times. While this assay offers superior sensitivity and the ability to multiplex, long incubation times and highly specialized equipment increase assay costs and limit sample throughput.

Work conducted by Han *et al.* also made use of a 96-well plate to develop a creative variation on traditional ELISA termed OLISA [56]. It is designed based on a detection antibody tethered to DNA through the incorporation of RNase H-mediated signal amplification. Using a fluorescence platform, the LOD for OLISA was

around 1 ng/ml using 100 µl of sample. This is comparable to the analogous ELISA, although slightly less sensitive. Detection antibodies employing different fluorophore/quencher pairs are employed that have independent spectral ranges for excitation and emission. This allows up to ten analytes to be interrogated in a single sample without signals arising from separate analytes interfering in the quantification of each compound. While this allows multiple analytes to be interrogated from a single sample, sensitivities are not improved relative to ELISA and incubation times, along with sample volumes, remain the same. Adaptations to the 96-well plate to allow nanoscale read volumes were achieved by the use of Siloam technology [58]. Incorporation of a spiral microchannel into each well of a microplate allows samples to be reduced to 5–10 µl and the washing to be reduced while mirroring the standard ELISA steps. The sensitivity of this commercial format is in line with that for OLISA and ELISA (around 1 pg/ml), but is accomplished in a time frame of 90 min and can accommodate multiple repeat samples to the same well to increase detection.

The static solid-support format has also been used to develop a fully automated ELISA on a portable disc-based format in work done by Lee *et al.* [59]. In this unique alternative to typical disc systems, fluid transfer occurs through ferrowax microvalves created using low-intensity laser light to melt paraffin wax embedded in iron oxide NPs. The paraffin valves allow the full integration of the immunoassay on-disc starting with a sample of whole blood. The assay is not limited in its number of steps as is typically seen with lab-on-a-disc systems where increasing spin speed is employed for sample transfers. With each disc having three identical units, multiple assays may be performed simultaneously in 30 min. The assay speed is afforded by disc rotation while sample is in the mixing chambers with reagents. This allows the assay to overcome time hurdles associated with diffusion-dependent incubations. Using 150 µl of whole blood, results show detection limits for anti-hepatitis B and hepatitis B antigen of 8.6 mIU/ml and 0.51 ng/ml, respectively. This represents LODs, comparable with ELISA using half the sample size and an assay time with a fourth of the duration. While this device is portable and disposable, assays are limited to the detection of three compounds simultaneously. This, coupled with the need to produce new devices for each assay, may result in high costs associated with fabrication. This assay shares similar qualities to the commercially available Gyrolab™, which is a completely integrated immunoassay system [60]. Using Gyrolab, 10-µl samples are loaded onto a special compact disk, which, through centrifugal force, is pushed into nanoscale channels containing streptavidin-coated bead columns used to trap the immunocomplex. While quantification may take place in 1 h, this format is limited to serial measurements and has high costs associated with specialized instrumentation and a single source of reagents.

As an alternative to expensive and complicated fabrication processes associated with many static immunoassays, a low-cost, microchip-based, fluorescent immunoassay was presented by Shao *et al.* for IgG detection [10]. The chip design is composed of four X-direction channels and one Y-direction channel to form four designated reaction zones (FIGURE 4). Areas between the zones were used as negative controls, where no obvious fluorescence was observed. Results show a LOD for IgG to be 5 ng/ml from a 10-µl sample, but this method requires long incubation times. These incubation times (1 h per step) were required as no agitation or mixing accompanied reaction steps, which were accomplished by diffusion after the initial channel filling. With times comparable to ELISA, this assay requires only a tenth of the sample volume to achieve analogous LODs. The same design could be used for the monitoring of multiple analytes with the possible integration of more designated reaction zones without additional technical complexity.

Although detection took place on a static printed array, Lian *et al.* describe the use of fluorescent NPs to produce a NP-labeled microarray [55]. In order to perform a multiplexed assay on the same slide, multiple blocks of capture antibodies were printed as subarrays. After incubation and wash steps occurring at room temperature as well as at 4°C for long time spans (2 h to overnight), select bioterrorism agents could be detected down to 10 pg/ml using 100 µl of sample over the entire array. The detection limits here represent roughly 100-fold improvements over fluorescent ELISA protocols used previously and require minimal sample use. However, equal or longer incubations are necessary, which would limit assay throughput and the ability to make serial analyses.

While most static solid-support assays require long incubation times to complete, Li *et al.* reported on a prefunctionalized PDMS microfluidic chip in an effort to produce an ultrafast heterogeneous immunoassay [7].

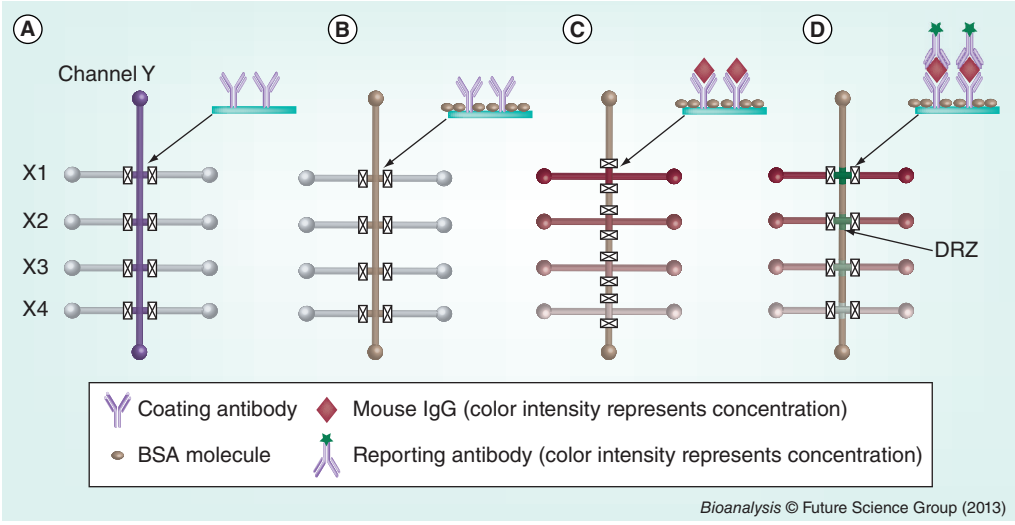

Figure 4. On-chip immunoassay protocol. Initially flowing coating antibody and blocking reagent through channel Y prepares the DRZs for the addition of analyte through the independent X channels. A reporting antibody can be delivered to each DRZ by addition through the Y channel, producing four independent DRZs, having negative controls present in the Y channel between reaction zones. The DRZs are prepared by **(A)** exposing channel Y to acoating antibody and **(B)** BSA as a blocking agent. **(C)** After sample addition through each X channel, **(D)** a reporting antibody can be delivered to each DRZ by addition through the Y channel. This produces four independent DRZs with negative controls present in the Y channel between reaction zones.
DRZ: Designated reaction zones.

Using an antigen–antibody reaction time of 5 min, the study found that blocking time had very little effect on the signal-to-noise ratio observed. This implies that nonspecific adsorption is reduced by short immunoreaction times. Results show a LOD for IgG of 600 ng/ml in an overall assay time of 19 min, while requiring only 10 nl of sample. This assay also offered the ability to quantify five analytes in parallel on a single chip. This assay boasts the advantage of completing analysis six-times faster than ELISA using a tenth of the sample volume. Since incubation steps are currently defined by diffusion, detection limits may be improved by the introduction of agitation during sample reactions. With the ability to more sensitively quantify many compounds in parallel using small sample volumes and short assay durations, this assay would be competitive with the most optimized formats currently employing mobile solid phases.

With an emphasis on assay sensitivity as opposed to rapid quantification, Lee *et al.* presented a sensitive total internal reflection fluorescence microscopy system for the detection of TNF-α on a nanoarray protein chip [24]. Using a homemade experimental system, TNF-α was successfully observed at a concentration of 0.13 fg/ml using a 50 µl sample. The total assay time took 2 h to complete, following the preparation of capture protein probe and sample. The assay affords a comparable assay duration to ELISA, but employs half the sample. Although it offers a highly sensitive assay that could be reasonably adapted to quantify multiple analytes, the assay requires sophisticated and specialized equipment.

While most static support immunoassay systems employ fluorescence detection, the Ju group investigated chemiluminescent immunoassay platforms for the near-simultaneous detection of two analytes, CEA and AFP [61,62]. In the first study, a modified glass tube with immobilized anti-CEA antibody was incubated with a mixture of the two antigens, their horseradish peroxidase-conjugated detection antibodies, and anti-AFP immobilized on paramagnetic particles [61]. After immunocomplexes were formed, AFP could be separated into an unmodified glass tube. Following separation, near simultaneous detection with the aid of an optical shutter could be performed. Results show detection limits for CEA and AFP of 0.6 and 0.89 ng/ml from 10 µl of sample, with negligible crossreactivity, respectively. A later study, based on a system of series wound immunosensing channels, was performed on the same target antigens [62]. With a procedure similar to

the one reported previously, the immunoassay could be completed in 27 min using 15 µl of sample. Here, the LODs for CEA and AFP were reduced to 0.39 and 0.41 ng/ml, respectively. Both assays afford the advantage of reduced assay duration and sample volume while maintaining comparable LODs to reference methods.

Many of the assays discussed in this section require relatively long incubation times and moderate sample volumes. These requirements are similar to those for microcantilevers, which offer novel detection modes using elegant physics [63,64]. While simple to operate and capable of attaining clinically relevant sensitivities (0.1 ng/ml), these methods require long incubations and large sample volumes (100–200 µl). Additionally, due to the detection platform they appear restricted in their ability to adapt to a multiplexed format, limiting their practical utility in diagnostic immunoassay applications.

While many of the methods discussed here have long incubation requirements, in cases where incubation times may be reduced through agitation or mixing, this format remains competitive with the on-chip assays utilizing magnetic solid supports. They offer the advantage of straightforward multiplexing and sensitive sample quantification, mostly without introducing complicated reaction processes or detection systems. While currently competitive with mobile solid-support formats, these assays rely heavily on detection through static fluorescence measurements. This limits their ability to be coupled with advanced signal processing mechanisms and may restrict their capacity to quantify target compounds at the low end of the physiological range.

Conclusion

Looking at the literature over this time span, a large number of publications focused on the use of a mobile solid phase, especially those utilizing magnetic microparticles or NPs. This emphasis evolved from the ease of manipulation through the introduction of magnetic forces that allowed for simple wash steps to be performed during the assay. In designs executed entirely on-chip, it also allowed flow conditions to be used for sample and reagent introduction, which drastically cuts overall assay time requirements. While this group of assays have primarily been evaluated in a single-analyte format, alterations allowing analysis of multiple compounds in parallel are straightforward and would not detract from sensitivity. In terms of attaining a fully optimized assay, fully on-chip immunoassays employing magnetic solid supports reduced sample size and time requirements while using simple detection methods and maintaining ease of use. The sensitivity achieved by methods with long incubation times could potentially be reached using convective mixing or slow flow rates to minimize the depletion layer surrounding the solid surface, all while maintaining rapid analysis. To tailor assays for clinical use, techniques must balance incubation durations and LODs. In addition, techniques employing a magnetic microparticle solid phase have demonstrated their compatibility with signal processing methods capable of improving detection limits to reach superior sensitivity and achieve a fully optimized immunoassay [4,5,28].

Another broad area of research is using fluorescent microbeads as a solid support, predominantly in the area of FMIA, which has been successfully used to detect up to ten analytes simultaneously with clinically acceptable levels of sensitivity. This approach is useful as it provides information about multiple analytes in the same sample volume as one traditional assay and could theoretically be used to detect up to 100 compounds in a single run. However, its ultimate limitation is the specificity of antibodies used, and the crossreactivity this produces between different targets. This could put a practical limitation on the number of compounds quantified simultaneously. Adaptions to testing for different analytes, while entirely possible, will require intensive assay development to ensure that singleplex assays for each compound give equivalent results to the multiplex assay used diagnostically. Additionally, due to the diffusion-mediated incubations, long assay durations are required to achieve acceptable LODs and this format is not readily capable of coupling to sophisticated signal processing methods. Nonetheless, in terms of practical immunoassay requirements, these tests can achieve the required level of multiplex capabilities and offer reasonable LODs (ng/ml to pg/ml) for most analytes.

Finally, the micro-mosaic assays offer the advantage of easy multiplexing and simple fabrication. They also produce LLODs, although they require moderate sample volumes and long incubation times resulting from the diffusion-mediated sample adsorption required using

this format. Similarly, the flow-through assays allow simple sample analysis. These assays also produce rapid results using moderate sample volumes. However, unlike the micro-mosaic assays they do not lend themselves easily to multiplexing.

Future perspective

While many of the assay formats described here were successful in improving upon one or more of the areas required for developing an optimized clinical test, none have been able to fully reach that mark. Today, the same issues challenging development of immunoassays remain. These issues consist of finding the appropriate balance between rapid analysis and sensitivity using techniques capable of coupling to signal processing methods, which may enhance detection limits. New techniques, in addition to consuming limited quantities of sample, should be currently capable of, or easily adaptable to, multiplexing without the requirement for highly specialized detection equipment. While various techniques have their specific advantages, a combination of aspects from multiple approaches appears to hold the greatest promise if a truly optimized assay is to be found.

With an increased understanding of reaction principles and conditions leading to superior sensitivity, immunoassay techniques continue to improve and progress toward optimization. Additionally, the now more familiar process of microfabrication enables the realistic implementation of many on-chip methods through large-scale photolithographic or injection mold production. In this arena, techniques will benefit from simple chip designs to ensure technical reproducibility. More development on this front over the next few years is poised to provide immunoassays well-tailored to their specific needs, which may be rapid analysis of samples taken on-site, or the ability to detect minute fluctuations in biomarkers over time indicative of disease states.

Financial & competing interests disclosure

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Executive summary

- Experimental immunoassays have failed to displace existing clinical tests and there is no available assay fully optimized for clinical testing that would require a test that is fast, affordable, able to multiplex, sensitive, easy to use and what uses low sample volumes.
- Emerging microimmunoassays can generally be grouped into four categories: those employing non-magnetized beads as a solid support, those employing magnetized beads as a solid support, assays where flow is used to produce signal and those employing a static solid support.
- Fluorescent microsphere immunoassay is a widely employed format utilizing non-magnetized particles to perform highly multiplexed analysis (theoretically up to 100 analytes from a single sample), but requiring specialized equipment and long incubation times.
- Magnetic microparticles have been used to construct assays both where off-chip (batch) incubation takes place before on-chip detection and those that occur fully on-chip.
- Although there are some instances where rapid assays completely performed on-chip have higher LODs, optimization of incubation times coupled with suitable signal processing is promising in attaining a highly sensitive assay.
- Assays producing signal through flow conditions have allowed short durations and turnover times, but are capable of detecting only one compound per assay and typically operate using a competitive assay format resulting in higher LODs.
- Many variations on the traditional 'static well' assay format have been developed utilizing a static solid support in which capture antibodies are patterned on polydimethylsiloxane, in capillary systems and antibody microarrays.
- Assays employing a static solid support are capable of straightforward multiplexing and sensitive analysis, but generally require moderate sample volumes and long incubation times.
- Many of the same issues that immunoassay development has faced in the past continue to be challenges and there is no single assay format that is optimized for comprehensive clinical testing.
- With the now familiar process of microfabrication, and an increased understanding of the reaction principles and conditions that can give superior results, techniques continue to improve.
- Over the next few years, more progress is poised to provide immunoassays capable of augmenting or replacing existing clinical tests, perhaps through a combination of aspects from multiple assays, to reach an optimal clinical test with those capable of rapidly detecting minute fluctuations in biomarker levels.

References

Papers of special note have been highlighted as:
- ▪ of interest
- ▪▪ of considerable interest

1 Engvall E, Jonsson K, Perlmann P. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. *Biochimica Biophysica Acta* 251, 427–434 (1971).

2 Hahn YK, Park JK. Versatile immunoassays based on isomagnetophoresis. *Lab Chip* 11, 2045–2048 (2011).

▪▪ **This assay format is adaptable both to highly sensitive detection and a wide dynamic range, making it a versatile platform for comprehensive analysis of biologically relevant targets throughout the plasma proteome.**

3 Ranzoni A, Sabatte G, van Ijzendoorn LJ, Prins MWJ. One-step homogeneous magnetic nanoparticle immunoassay for biomarker detection directly in blood plasma. *ACS Nano* 6(4), 3134–3141 (2012).

▪ **This assay offers a user-friendly platform and generates sensitive and rapid results for a single target.**

4 Mahanti P, Taylor T, Hayes MA, Cochran D, Petkus MM. Improved detectability and signal strength for rotating phase fluorescence immunoassays through signal processing. *Analyst* 136, 365–373 (2011).

▪▪ **Coupling immunoassay techniques to novel signal processing methods could allow extension of detection limits without alterations to data capture, which would allow rapid assay methods to be developed without sacrificing sensitivity.**

5 Chang B, Gray P, Piltch M *et al.* Surround optical fiber immunoassay (SOFIA): an ultra-sensitive assay for prion protein detection. *J. Virol. Methods* 159, 15–22 (2009).

▪▪ **Through the use of specialized equipment, coupled to signal processing, the ultra-sensitive detection of proteins is possible (~10 attogram detection limit).**

6 Ziegler J, Zimmermann M, Hunziker P, Delamarche E. High-performance immunoassays based on through-stencil patterned antibodies and capillary systems. *Anal. Chem.* 80, 1763–1769 (2008).

7 Li P, Abolmaaty A, D'Amore C, Demming S, Anagnostopoulos C, Faghri M. Development of an ultrafast quantitative heterogeneous immunoassay on pre-functionalized poly(dimethylsiloxane) microfluidic chips for the next-generation immunosensors. *Microfluid Nanofluidics* 7, 593–598 (2009).

8 Peyman SA, Iles A, Pamme N. Mobile magnetic particles as solid-supports for rapid surface-based bioanalysis in continuous flow. *Lab Chip* 9, 3110–3117 (2009).

9 Sasso LA, Under A, Zahn JD. Autonomous magnetically actuated continuous flow microimmunofluorocytometry assay. *Microfluid Nanofluidics* 9, 253–265 (2010).

10 Shao G, Wang J, Li Z, Saraf L, Wang W, Lin Y. Poly(dimethylsiloxane) microchip-based immunoassay with multiple reaction zones: toward on-chip multiplex detection platform. *Sens. Actuators B Chem.* 159, 44–50 (2011).

▪ **Low-cost analysis of samples is possible through the scaling of current experimental formats, allowing highly parallel analysis without complicating fabrication.**

11 Do J, Ahn CH. A polymer lab-on-a-chip for magnetic immunoassay with on-chip sampling and detection capabilities. *Lab Chip* 8, 542–549 (2008).

12 Langenhorst RJ, Lawson S, Kittawornrat A *et al.* Development of a fluorescent microsphere immunoassay for detection of antibodies against porcine reproductive and respiratory syndrome virus using oral fluid samples as an alternative to serum-based assays. *Clin. Vacc. Immunol.* 19, 180–189 (2011).

13 Lawson S, Lunney J, Zuckermann F *et al.* Development of an 8-plex Luminex® assay to detect swine cytokines for vaccine development: assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. *Vaccine* 28, 5356–5364 (2010).

14 Cao Z, Li H, Lau C, Zhang Y. Cross-talk-free simultaneous fluoroimmunoassay of two biomarkers based on dual-color quantum dots. *Analytica Chimica Acta* 698, 44–50 (2011).

15 Zimmermann M, Hunziker P, Delamarche E. Autonomous capillary system for one-step immunoassays. *Biomed. Microdevices* 11, 1–8 (2009).

16 Lacharme F, Vandevyver C, Gijs MAM. Magnetic beads retention device for sandwich immunoassay: comparison of off-chip and on-chip antibody incubation. *Microfluid Nanofluidics* 7, 479–487 (2009).

▪▪ **Utilizing magnetic microbeads in fully on-chip immunoassays allows rapid analysis utilizing minimal sample volumes and producing clinically relevant sensitivities.**

17 Dupont EP, Labonne E, Vandevyver C, Lehmann U, Charbon E, Gijs MAM. Monolithic silicon chip for immunofluorescence detection on single magnetic beads. *Anal. Chem.* 82, 49–52 (2010).

18 Lacharme F, Vandevyver C, Gijs MAM. Full on-chip nanoliter immunoassay by geometrical magnetic trapping of nanoparticle chains. *Anal. Chem.* 80, 2905–2910 (2008).

19 Sivagnanam V, Song B, Vandevyver C, Gijs MAM. On-chip immunoassay using electrostatic assembly of streptavidin-coated bead micropatterns. *Anal. Chem.* 81, 6509–6515 (2009).

20 Sivagnanam V, Bouhmad A, Lacharme F, Vandevyver C, Gijs MAM. Sandwich immunoassay on a microfluidic chip using patterns of electrostatically self-assembled streptavidin-coated beads. *Microelectron. Eng.* 86, 1404–1406 (2009).

21 Pfaunmiller E, Moser AC, Hage DS. Biointeraction analysis of immobilized antibodies and related agents by high-performance immunoaffinity chromatography. *Methods* 56, 130–135 (2012).

22 Schiel JE, Tong Z, Sakulthaew C, Hage DS. Development of a flow-based ultrafast immunoextraction and reverse displacement immunoassay: analysis of free drug fractions. *Anal. Chem.* 83, 9384–9390 (2011).

▪ **If there is a single target of interest, rapid immunoassays can be performed with very short turnover times using reverse displacement immunoassay in a competitive assay format.**

23 Pesce AJ, Michael JG. Artifacts and limitations of enzyme-immunoassay. *J. Immunol. Methods* 150, 111–119 (1992).

24 Lee S, Cho NP, Kim JD, Jung H, Kang SH. An ultra-sensitive nanoarray chip based on single-molecule sandwich immunoassay and TIRFM for protein detection in biologic fluids. *Analyst* 134, 933–938 (2009).

25 Vignali DAA. Multiplexed particle-based flow cytometric assays. *J. Immunol. Methods* 243, 243–255 (2000).

26 Allinson JL. Automated immunoassay equipment platforms for analytical support of pharmaceutical and biopharmaceutical development. *Bioanalysis* 3, 2803–2816 (2011).

27 Verch T, Bakhtiar R. Miniaturized immunoassays: moving beyond the microplate. *Bioanalysis* 4, 177–188 (2012).

28 Hayes MA, Petkus MM, Garcia AA, Taylor T, Mahanti P. Demonstration of sandwich and competitive modulated supraparticle fluoroimmunoassay applied to cardiac protein biomarker myoglobin. *Analyst* 134, 533–541 (2009).

29 Watson DS, Reddy SM, Brahmakshatriya V, Lupiani B. A multiplexed immunoassay for detection of antibodies against avian influenza virus. *J. Immunol. Methods* 340, 123–131 (2009).

30 Kellar KL, Douglass JP. Multiplexed microsphere-based flow cytometric immunoassays for human cytokines. *J. Immunol. Methods* 279, 277–285 (2003).

31 Clavijo A, Hole K, Li M, Collignon B. Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle. *Vaccine* 24, 1693–1704 (2006).

32 Ravindran R, Khan IH, Krishnan VV *et al.* Validation of multiplex microbead immunoassay for simultaneous serodetection of multiple infectious agents in laboratory mouse. *J. Immunol. Methods* 363, 51–59 (2010).

33 Svateck RS, Shah JB, Xing J *et al.* A multiplexed, particle-based flow cytometric assay identified plasma matrix metalloproteinase-7 to be associated with cancer-related death among patients with bladder cancer. *Cancer* 116, 4513–4519 (2010).

34 Smith J, Samons D, Robertson S, Biagini R, Snawder J. Measurement of multiple drugs in urine, water, and on surfaces using fluorescence covalent microbead immunosorbent assay. *Toxicol. Mech. Methods* 20, 587–593 (2010).

35 Kuriakose T, Hilt DA, Jackwood MW. Detection of avian influenza viruses and differentiation of H5, H7, N1, and N2 subtypes using a multiplex microsphere assay. *Avian Dis.* 56, 90–96 (2012).

36 Pochechueva T, Chinarev A, Spengler M *et al.* Multiplex suspension array for human anti-carbohydrate antibody profiling. *Analyst* 136, 560–569 (2011).

37 Ji XH, Cheng W, Guo F *et al.* On-demand preparation of quantum dot-encoded microparticles using a droplet microfluidic system. *Lab Chip* 11, 2561–2568 (2011).

38 Talat N, Shahid F, Perry S *et al.* Th1/Th2 cytometric bead array can discriminate cytokine secretion from endogenously activated cells in pulmonary disease, recent and remote infection in tuberculosis. *Cytokine* 54, 136–143 (2011).

39 Bin Z, Ke W, Jian J *et al.* Quantitative determination of deoxynivalenol (DON) using the amplified luminescent proximity homogeneous assay (AlphaLISA). *Food Anal. Methods* 4, 228–232 (2011).

40 Cauchon E, Liu S, Percival MD *et al.* Development of a homogeneous immunoassay for the detection of angiotensin I in plasma using AlphaLISA acceptor beads technology. *Anal. Biochem.* 388(1), 134–139 (2009).

41 Li Z, Zhang QY, Zhao LX *et al.* Micro-plate magnetic chemiluminescence immunoassay and its applications in carcinoembryonic antigen analysis. *Sci. China Chem.* 53, 812–819 (2010).

42 Sista RS, Eckhardt AE, Srinivasan V, Pollack MG, Palanki S, Pamola VK. Heterogeneous immunoassays using magnetic beads on a digital microfluidic platform. *Lab Chip* 8, 2188–2196 (2008).

43 Kim JI, Wang C, Kuizon S *et al.* Simple and specific detection of abnormal prion protein by a magnetic bead-based immunoassay coupled with laser-induced fluorescence spectrofluorometry. *J. Neuroimmunol.* 158, 112–119 (2005).

44 Proczek G, Gassner AL, Busnel JM, Girault HH. Total serum IgE quantification by microfluidic ELISA using magnetic beads. *Anal. Bioanal. Chem.* 402, 2645–2653 (2012).

45 Piao Y, Jin Z, Lee D *et al.* Sensitive and high-fidelity electrochemical immunoassay using carbon nanotubes coated with enzymes and magnetic nanoparticles. *Biosens. Bioelectron.* 26, 3192–3199 (2011).

46 Soh N, Tanaka M, Hirakawa K *et al.* Sequential injection immunoassay for environmental measurements. *Anal. Sci.* 27, 1069–1076 (2011).

47 Gao D, Li HF, Guo GS, Lin JM. Magnetic bead based immunoassay for enumeration of CD4+ T lymphocytes on a microfluidic device. *Talanta* 82, 528–533 (2010).

48 Chen H, Abolmatty A, Faghri M. Microfluidic inverse phase ELISA via manipulation of magnetic beads. *Microfluid Nanofluidics* 10, 593–605 (2011).

49 Squires TM, Messinger RJ, Manalis SR. Making it stick: convection, reaction and diffusion in surface-based biosensors. *Nat. Biotechnol.* 26, 417–426 (2008).

50 Song SY, Han YD, Kim K, Yang SS, Yoon HC. A fluoro-microbead guiding chip for simple and quantifiable immunoassay of cardiac troponin I (cTnI). *Biosens. Bioelectron.* 26, 3818–3824 (2011).

51 Afshar R, Moser Y, Lehnert T, Gijs MAM. Three-dimensional magnetic focusing of superparamagnetic beads for on-chip agglutination assays. *Anal. Chem.* 83, 1022–1029 (2011).

52 Fu Z, Shao G, Wang J, Lu D, Wang W, Lin Y. Microfabricated renewable beads-trapping/releasing flow cell for rapid antigen–antibody reaction in chemiluminescent immunoassay. *Anal. Chem.* 83, 2685–2690 (2011).

53 Liu Y, Wang H, Huang J, Yang J, Liu B, Yang P. Microchip-based ELISA strategy for the detection of low-level disease biomarker in serum. *Anal. Chim. Acta* 650, 77–82 (2009).

54 Sinha A, Ganguly R, Puri IK. Numerical investication of flow-through immunoassay in a microchannel. *J. Appl. Phys.* 107(3), 034907 (2010).

55 Lian W, Wu D, Lim DV, Jin S. Sensitive detection of multiplex toxins using antibody microarray. *Anal. Biochem.* 401, 271–279 (2010).

56 Han KC, Ahn DR, Yang EG. An approach to multiplexing an immunosorbent assay with antibody-oligonucleotide conjugates. *Bioconjugate Chem.* 21, 2190–2196 (2010).

57 Ruckstuhl T, Winterflood CM, Seeger S. Supercritical angle fluorescence immunoassay platform. *Anal. Chem.* 83, 2346–2360 (2011).

58 Kai J, Puntambekar A, Sehy D, Brescia P, Banks P. Amplifying Immunoassay Sensitivity. *Gen. Engineer. Biotechnol. News* 31, 26–27 (2011).

59 Lee BS, Lee JN, Park JM *et al.* A fully automated immunoassay from whole blood on a disc. *Lab Chip* 9, 1548–1555 (2009).

60 Roman J, Qiu J, Dornadula G *et al.* Application of miniaturized immunoassays to discovery pharmacokinetic bioanalysis. *J. Pharm. Toxic. Methods* 63, 227–235 (2011).

61 Yang Z, Liu H, Zong C, Yan F, Ju H. Automated support-resolution strategy for a one-way chemiluminescent multiplex immunoassay. *Anal. Chem.* 81, 5484–5489 (2009).

62 Yang Z, Zong C, Yan F, Ju H. Automated chemiluminescent dual-analyte immunoassay based on resolved immunosensing channels. *Talanta* 82, 1462–1467 (2010).

63 Xue C, Zhao H, Liu H *et al.* Development of sulfhydrylated antibody functionalized microcantilever immunosensor for taxol. *Sens. Actuators B Chem.* 156(2), 863–866 (2011).

64 Tan W, Huang Y, Nan T *et al.* Development of protein A functionalized microcantilever immunosensors for the analysis of small molecules at parts per trillion levels. *Anal. Chem.* 82, 615–620 (2010).

■ Website

101 RNase H1Antibody (5D10). www.novusbio.com/H00246243-M01