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Automated processing integrated with a microflow cytometer for pathogen detection in clinical matrices

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ABSTRACT

A spinning magnetic trap (MagTrap) for automated sample processing was integrated with a microflow cytometer capable of simultaneously detecting multiple targets to provide an automated sample-to-answer diagnosis in 40 min. After target capture on fluorescently coded magnetic microspheres, the magnetic trap automatically concentrated the fluorescently coded microspheres, separated the captured target from the sample matrix, and exposed the bound target sequentially to biotinylated tracer molecules and streptavidin-labeled phycoerythrin. The concentrated microspheres were then hydrodynamically focused in a microflow cytometer capable of 4-color analysis (two wavelengths for microsphere identification, one for light scatter to discriminate single microspheres and one for phycoerythrin bound to the target). A three-fold decrease in sample preparation time and an improved detection limit, independent of target preconcentration, was demonstrated for detection of *Escherichia coli* 0157:H7 using the MagTrap as compared to manual processing. Simultaneous analysis of positive and negative controls, along with the assay reagents specific for the target, was used to obtain dose–response curves, demonstrating the potential for quantification of pathogen load in buffer and serum.

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1. Introduction

To move lab-on-a-chip (LOC) devices out of the lab and into the hands of a user usually requires integration of multiple system components. Ideally, a user would simply put a sample into a system and obtain a reliable, actionable answer (Gervais et al., 2011; Gubala et al. 2012; Ligler 2009; Yager et al., 2006). To analyze complex sample matrices (e.g. clinical fluids, environmental samples, food and beverages) with high sensitivity for targets of interest, sample processing components are necessary that are in themselves portable and automated, as well as compatible with the portable LOC analytical component. In a recent, very comprehensive review of microfluidic devices for point-of-care immunodiagnostics, Gervais et al. (2011) define an ideal 1$ integrated biosensor for multiplexed diagnostics that includes an input for a clinical sample, target preconcentration, microarrays of immobilized recognition molecules, sample processing reagents, mixers, valves, pumps, optics and electronics for data collection, analysis, and transmission; such a device does not yet exist, but we predict that it will (at a higher cost).

Automated sample processing using affinity reactions is generally more flexible with regard to sample type and reaction temperature, and the reagents are usually more stable than procedures employing amplification enzymes. Sample processing with affinity reagents usually involves target capture and separation from the sample matrix, followed by incubation with a series of additional binding reagents which increase the signal generated by each target to achieve the required sensitivity. Critical issues for efficiency of sample processing with affinity reagents include binding constants of recognition molecules (especially after immobilization or labeling), mixing to eliminate the formation of depletion layers at surfaces (especially with viscous samples), binding specificity of all reagents, and nonspecific binding. Most automated affinity processing systems are coupled directly to the analytical component (Gervais et al., 2011; McKenzie et al., 2009). Lateral flow immunoassays are a familiar example. However, a variety of affinity processing systems coupled with immunoassays have been reported using fluid flow driven by centrifugal force into an optically interrogated chamber (Gorkin et al., 2012; Lee et al., 2009; Peytavi et al., 2005) or by pressure-driven flow (Jokerst et al., 2010; Lafleur et al., 2012; McKenzie et al., 2009) over immobilized antibody arrays. In these reports, the higher the number of processing steps, the more complicated the device becomes. Interestingly, the degree of multiplexing does not generally have a major impact on the

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complexity of the processing, but is limited primarily by the cross-reactivity of the reagents used for a particular multiplexed assay.

The first step in automating sample preparation is usually target capture, preferably with target preconcentration out of larger sample volumes into μL–nL volumes, in order to take advantage of microfluidic systems that can most efficiently process small volumes. Immunomagnetic capture is a well-established technology using antibody-coated magnetic microspheres to pull target out of large volumes of sample (Palecek and Fojta, 2007). In addition to antibodies, magnetic microspheres have been coated with oligonucleotides and other capture molecules for target concentration. The advantages of using immunomagnetic microspheres for capture include stability during storage, ease of manipulation, and flexibility for use with variable sample types and volumes. The disadvantages relate more generally to use in downstream processing; in many cases, the target must be removed from the microspheres for processing and/or analysis. Kwon et al. (2008) bypassed the need to release the target from the clustered magnetic microspheres by measuring the photoinduced release of fluorescent eTags from the tracer antibodies in complexes of microspheres, capture antibody, target, and tracer antibody, but this approach requires a light-emitting diode (LED) for processing, as well as a magnet, valves and pumps, increasing the complexity of manipulations required prior to the analysis. In most of the systems reported to date, the magnetic microspheres are pulled together with a fixed magnet; this clumping can generate aggregates or sequester the target, reducing the efficiency of subsequent reagent binding or analysis. Two systems have been reported to avoid this aggregation; both use spinning magnets that pull the microspheres continuously upstream during processing. In the processing component reported by Anderson and colleagues (Anderson et al., 2009), proteins are captured on the magnetic microspheres that are pulled upstream in a horse-shoe shaped tube, concentrated out of the sample, and then trypsinized to remove peptides from the magnetic microspheres for mass spectrometry. In the MagTrap used here, the spinning magnets pull the magnetic microspheres upstream and side-to-side during processing in a microchannel, there is no target release step, and the microspheres are released from the magnetic field for direct introduction into the analytical device (Howell et al., 2011; Verbarg et al., 2012).

Automation of sample processing and flow cytometry has been explored using large flow cytometry systems, primarily for increasing throughput. Usually, the samples are still processed (manually or robotically) in a 96-well plate and automatically sipped into a cytometer. The autonomous pathogen detection system automatically processes aqueous samples from an air collector to test for biowarfare agents using a Luminex flow cytometer and off-the-shelf fluidic components in a large free-standing system (Dzenitis and Makarewics, 2010; Hindson et al., 2005). Another interesting example of an integrated sample processing-analytical system has just been reported by Kuystermans et al. (2012) which combines the commercial FlowCytoPrep device (MSP Corp, MN) with a benchtop cytometer to monitor proliferation of cells in culture in an automated process including cell fixation and staining. Large cytometers such as the CytoBot and CytoSense have also been used to evaluate a continuous stream of algae underwater, but other than filtration through a screen, there is no sample processing involved. To our knowledge, the first report of an automated microflow cytometer was provided by James Leary's group in 2012 (Maleki et al., 2012). This whole blood analyzer labeled white cells or tumor cells with immuno-quantum dots for identification and/or immunomagnetic microspheres for sorting. The device included a micro-mixer, separation system, LED, avalanche photodiode, and electronics that operated on a 9-volt battery. Although that report focused primarily on the device rather than application data, the authors provided an elegant proof-of-principle experiment for sorting and counting CD45-positive cells.

Coded microspheres were developed in order to minimize the complexity of the optics required for multiplexed analyses (Walt, 2000). Microspheres coded with different amounts of multiple fluorophores are now widely used as substrates for multiplexed immunoassays that can be processed efficiently and analyzed using imaging or flow cytometry. We developed a microflow cytometer for analyzing multiplexed immunoassays based on a four-color analysis of fluorescently coded microspheres (Golden et al., 2010; Kim et al., 2009). Ten-plex assays in spiked buffer have demonstrated limits of detection comparable to benchtop commercial systems in assays using the same reagents, with sensitivities as low as 10 pg/ml for toxins and 10^4 cells/ml for bacteria.

In all prior reports using the microflow cytometer, sample processing was performed manually in tubes or microtiter plates. In this report, we integrate the microflow cytometer with the MagTrap sample processing component. The MagTrap does more than just trap microspheres on the side of a microfluidic channel. Spinning magnets under the channel pull the microspheres (including those with captured target) upstream against the flow and side-to-side in the channel to both collect and concentrate the microspheres as the sample is introduced, and to expose the microspheres sequentially to the reagents (Verbarg et al., 2012). Then the rotation of the magnets is reversed, and the concentrated, but not aggregated, microspheres are released for analysis. Here we (1) improve the performance of the microflow cytometer using a streamlined fiber optic configuration, (2) connect the output from the MagTrap directly into the microflow cytometer, (3) evaluate the immunoassay results for the detection of *Escherichia coli* and (4) compare the results to the same assays performed manually. Additionally, we demonstrate that automated immunoassays can be performed in a clinical sample.

2. Material and methods

2.1. MagTrap design and function

The spinning magnetic trap, or "MagTrap", reported in this paper combines the advantages of immunomagnetic target capture with dynamic manipulation of the magnetic microspheres inside a microfluidic channel. Permanent magnets were arranged on a rotating wheel and positioned directly beneath the microchannel, as shown in Fig. 1. The microchannel was hot embossed in polymethyl methacrylate (PMMA) from a trapezoidal mould (500 μm top width, 355 μm bottom width, 125 μm height). The magnets were placed under the narrow part of the trapezoidal channel so that trapping of the microspheres in the corners was minimized. Details of the rotating MagTrap design and function, as well as the microchannel embossing and bonding, have been previously reported (Verbarg et al. 2012).

During sample processing, the magnets rotate clockwise under the microchannel, collecting the immunomagnetic microspheres without aggregation and moving them against the flow, as well as from one side of the channel to the other. When the leading magnet rotates away from the channel, the microspheres are briefly released into the flow stream and then trapped by the next magnet. Movement of the microspheres against the flow of the incubation reagent increases the interaction of the microspheres with reagents in the flow stream. Reversal of the magnets' rotation sweeps the microspheres downstream. When the