LASER-ACTUATED CENTRIFUGO-PNEUMATIC FLOW CONTROL TOWARDS 'SAMPLE-TO-ANSWER' INTEGRATED DETECTION OF MULTI-MARKER PANELS AT THE POINT-OF-CARE

Rohit Mishra[1], Glen Reilly[1], Matthew Agnew[1], Alexander Garvey[1], Ciaran Rogers[1], Elaine Andrade[1], Hui Ma[2], Sean Fitzgerald[2], Julia Zapatero[2], Richard O'Kennedy[2, 3] and Jens Ducrée[1]

[1]FPC@DCU – Fraunhofer Project Centre for Embedded Bioanalytical Systems at Dublin City University, Glasnevin, Dublin 9, Ireland.

[2]School of Biotechnology, Dublin City University, Dublin 9, Ireland.

[3] Research Complex, Hamad Bin Khalifa University, Doha, Qatar.

ABSTRACT

Point-of-care diagnostics devices require user-friendly automation of multi-step / multi-reagent bioanalytical protocols. We demonstrate, for the first time, Laser-actuated, Centrifugo-Pneumatic Valves (LCPVs) which open on-demand by ablating a remotely connected polymeric membrane with an instrument-based laser. Due to its small footprint, isolation of radiation-induced heating from the biological fluids and independence of radial position & contact-angle, the novel LCPV technology enables large–scale integration of flexibly customizable and operationally robust centrifugal microfluidic "Lab-on-a-Disc" (LoaD) systems. As a pilot application, we demonstrate the detection of Fatty Acid Binding Protein and Myeloperoxidase, both potential biomarkers used for cardiovascular disease (CVD) determination.

INTRODUCTION

Nowadays centrifugal microfluidic technologies have solidly established in the area of decentralized bioanalytical testing, in particular for sample-to-answer automation of biomedical point-of-care applications. At the fluidic backbone of these LoaD systems, liquids experience the rotationally controlled centrifugal field in a network of microstructures and channels representing Laboratory Unit Operations (LUOs), such as metering & mixing, connected by interspersed valves [1, 2]. Striking benefits of LoaD systems are their inherent capability for centrifugal pumping, agitation and particle sedimentation based on a simple spindle motor running without error prone pneumatic interfaces. Various bioanalytical protocols for detecting/ small molecules, proteins, antibodies, nucleic acids, cells, bacteria and other bioparticles have been successfully implemented [3].

A range of normally-closed flow control schemes have been generated for centrifugal microfluidic platforms [2-6]. Such valves are often based on capillary stops and siphoning principles. Also sacrificial barrier materials such as wax are used; however, these valves tend to be difficult to manufacture at the required accuracy and reliability while keeping cost low [7]. Other flow barriers [8, 9] directly block liquid flow until opened by high intensity electromagnetic irradiation, e.g. a laser. This high-energy interaction may lead to chemical modification or contamination of the adjacent liquids.

The burst frequencies of so-called centrifugo-pneumatic valves that are initially sealed by dissolvable films (DF) [10-12] feature a large spread in the definition of release frequencies, hence limiting the number of independently controlled operations that can be integrated in the same disc. Good progress has been made over recent years by pneumatically mediated cascading of such DF valves to allow comprehensive automation of more complex multi-step, multi-reagent bioanalytical protocols [11]. However, the large footprint of these valves is still an issue when moving towards higher-level integration of fluidic networks on the LoaD.

We present here a new type of laser-actuated centrifugo-pneumatic valves (LCPVs). In their initial state, liquid is prevented from entering the valve by compression of entrapped gas. Exposure of the control film (CF) by an instrument-based laser vents the pneumatic chamber, thus allowing the liquid to wet and subsequently exit through the DF.

There are several advantages of these LCPVs: Compared to direct membrane blocking of the flow, the thermal impact is localized at a distal CF, and thus well insulated from the liquid to avoid its potential damage and contamination. Furthermore, their "digital" nature renders the flow control mechanism largely independent of its radial position, manufacturing tolerances and capillary effects. The small footprint facilitates use of the LCPVs a good candidate for high-density process integration of fluidic networks. Additionally, focusing of the 'contact-free' laser actuation enables selective opening of CFs that are vertically distributed across stacked disc layers through focusing on their respective optical plane.

SYSTEM DESIGN

We demonstrate here the design, operation and application of the new LCPV technology based on laser perforation of a polymeric barrier films. In their default, normally-closed state, these thin CFs seal a pneumatic chamber created between the incoming fluid reservoir / microchannel and the exit channel sealed with a DF. The 3D architecture (Fig. 1) is constituted by structured PMMA discs bonded by interspersed layers of pressure sensitive adhesive (Fig. 2) [11]. As they are either transparent or exhibit a local 'cut-out', the CFs in different layers can be addressed by vertical translation of the laser (Fig. 2B). This configuration offers wide flexibility of the placement of the LCPV as long as a pneumatic chamber is still formed between the CF, the DF and the incoming liquid under the impact of the centrifugal field.

Figure 3 illustrates the functional principle of the LCPV. At the outset, liquid in the reservoir is held back from wetting the DF on the exit channel under the impact of the centrifugo-pneumatic compression of the trapped gas pocket according to Boyle's law. Once the laser

pierces the thin CF in about ~1 s, the pneumatic chamber is vented and the liquid can proceed to wet and thus open the DF tab that so far blocked the exit channel below.

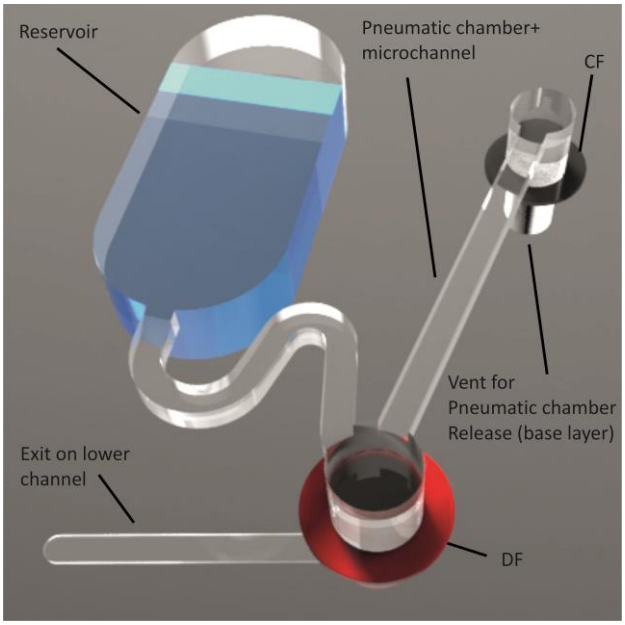

Figure 1: Basic LCPV configuration. While the CF and the DF are intact, the incoming water from the reservoir (in blue) entraps a gas pocket between the LCPV, DF and the water (labelled Pneumatic chamber + microchannel). When the CF is laser-ablated on-demand, the pneumatic chamber is vented to the atmosphere via the lower vents in the base layer of a multi-layered disc. Driven by the centrifugal force, the water then proceeds to wet and dissolve the DF to let the liquid proceed to the exit channel.

Thus, LCPVs provide a simple, on-demand, small-footprint technique that also allows release of liquids widely independent of their radial position and surface wettability. This allows for higher-level system integration and parallelization of multi-sample and multi-analyte assay protocols. We demonstrate this by fully automating the entire sample handling protocol for detecting a potential multi-marker panel of CVD diagnostics.

BIOMARKER ASSAY AUTOMATION

CVD comprises a wide range of diseases and abnormal conditions affecting the heart or coronary vessels. The development of a rapid, sufficiently sensitive and specific tool for diagnostics and early-stage prognostics is highly desirable [13, 14]. So far, commercially available cardiac biomarker POC devices have focused mainly on the detection of myoglobin and cardiac troponins [15]. However, instead of relying on a sole marker, it is now suggested that biomarker panels featuring a combination of select markers may significantly improve diagnosis of CVD [16].

We now employ an LCPV-controlled LoaD system to automate the liquid handling protocol at the backbone of an ELISA including all the steps from initialt plasma extraction to incubation, buffer washes and the final enzymatic reaction for signal generation (Fig. 4). This technology will pave the way towards implementation of a sample-to-answer device for the point-of-care detection of at least three cardiac biomarkers and corresponding controls (we demonstrate two here). The compact valve design even allows reserving space for further parameters.

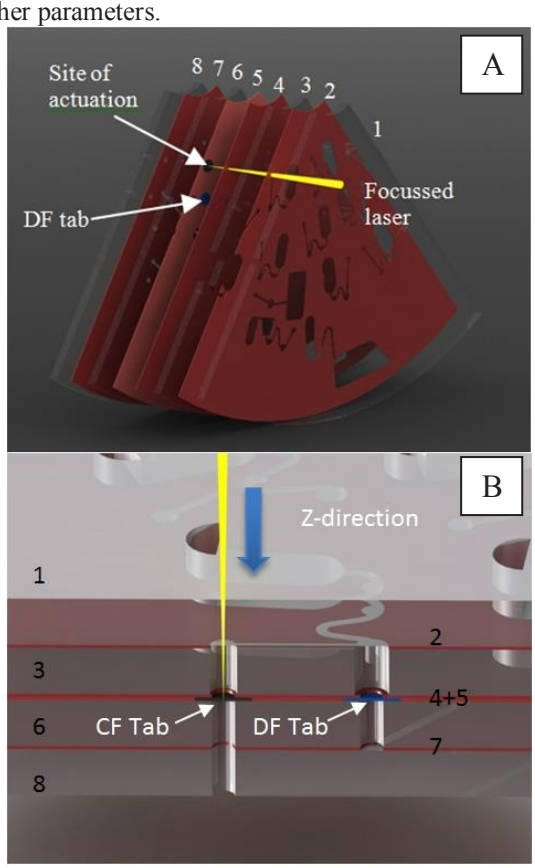

Figure 2: A) The laser actuates through multiple layers of PMMA and PSA (as long as they are transparent or allow modification for light to pass through) allowing wide range X-Y actuation. B) The focal point of the laser can be moved in order to actuate several membranes along the vertical (Z) direction, thus allowing to reach any deeply recessed valve in the system. (1-8 are the layers of the LoaD with red layers designating pressure sensitive adhesive and the transparent thick layers being PMMA plastic)

For the current study, two CVD biomarkers, Heart-Type Fatty Acid Binding Protein (hFABP) and Myeloperoxidase (MPO) are detected using a competitive ELISA format. The concentration of hFABP is known to rise during cardiac necrosis and is thus a sensitive indicator of ischemia in acute myocardial infarction [17]. Detection of MPO within a hospital setting was shown to be a potential risk predictor for primary cardiac incidents [18].

EXPERIMENTAL PROTOCOL

Benchtop Competitive ELISA:

Half the area of 96-well plates (Fisher, Ireland) were coated with 30 µL per well of 2.5 µg mL^{-1} hFABP or 2 µg mL^{-1} MPO (AMS Biotechnologies, UK) at 4°C overnight. Plates were blocked with 60 µL per well of 5% (w/v) Milk Marvel in Phosphate Buffered Saline Tween-

20 (PBST) at 37°C for one hour. The plate was washed with Phosphate Buffered Saline (PBS) (60 µL / well). To carry out the competitive ELISA, a range of concentrations of FABP (0-500 ng mL^{-1}) or MPO (0-2000 ng mL^{-1}) were prepared and mixed with a fixed concentration of antiFABP scFv (0.26 µg mL^{-1}) and anti-MPO (0.03 µg mL^{-1}) scFv. This mixture (30 µL / well) was incubated at room temperature for 10 min. A negative control, consisting of 30 µL / well of 'FABP-free' human sera and MPO free human sera was incorporated. The plate was washed with PBS (60 µL / well). Then HRP-labeled anti-HA antibody (1:1,000, 30 µL/well) was added (the scFv have a HA tag) and incubated at room temperature for 10 min. The plate was washed with PBS (60 µL / well). TMB (0.4 g L^{-1}, 30 µL / well), the HRP substrate, was added and incubated at room temperature for 2 min. The reaction was stopped by addition of 30 µL of 1M HCl per well. The absorbance of the wells was read at 450 nm using a Safire 2 plate reader.

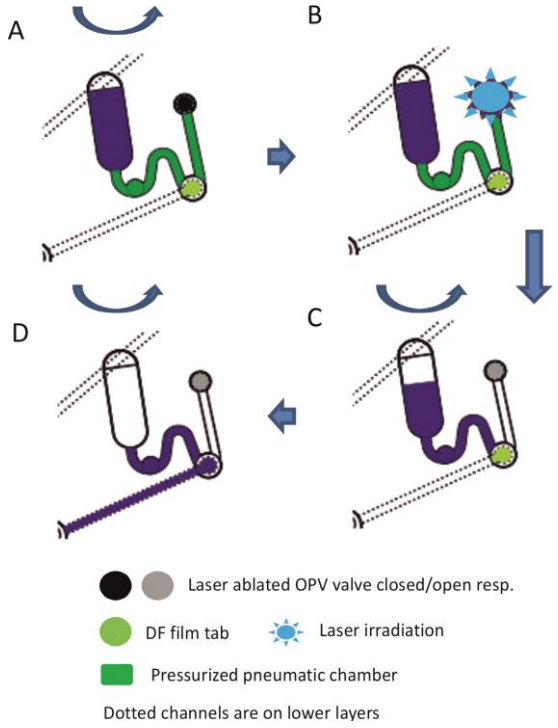

Figure 3: Release mechanism for the opto-pneumatic valves. A. The reservoir is loaded with aqueous solution. A pressurized pneumatic chamber is created (in green) between the CF and the DF which prevents the liquid from priming the exit channel and wetting of the DF. B. Laser irradiation (415 nm, ~1 sec) releases the pressure in the pneumatic chamber via a vent on the base layer. C. The microchannel is immediately primed and wets the DF. D. The dissolution of the DF releases the liquid into the lower channel en route to the next chamber and the reservoir is emptied by increasing the spin frequency.

LoaD Competitive ELISA:

The disc is a hybrid assembly of three layers of poly-methyl methacrylate (PMMA), a base layer of polystyrene (for immobilization) along with four layers of pressure sensitive adhesive (Adhesives Research, Limerick, Ireland). The manufacturing method and assembly have been described elsewhere [11, 19].

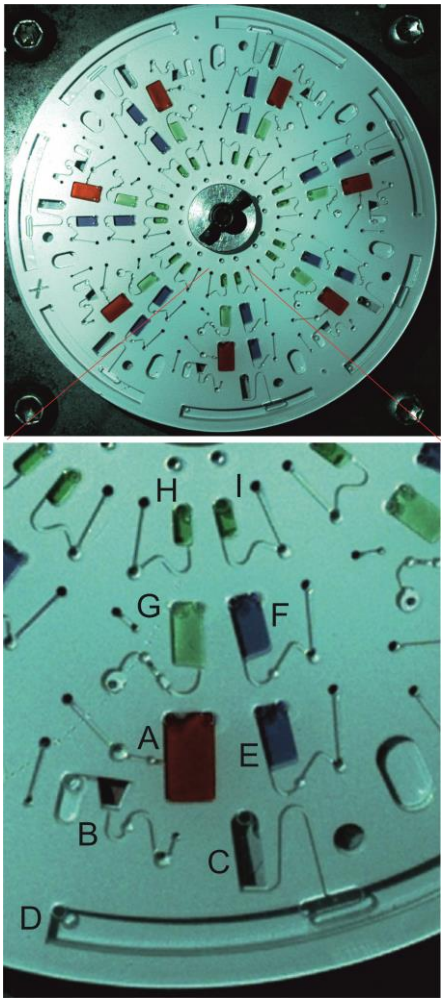

Figure 4: Multi-marker LoaD with seven sections, liquid chambers and LCPV valves. Chamber: A-Blood, B-Plasma metering, C-Incubation and detection, D-Waste, E-First Buffer Wash, F-Second Buffer Wash, G- Stop Acid, H-Secondary Antibody, I-TMB colorimetric substrate.

To prepare the discs, the incubation chambers were coated with 30 µL per chamber of 5 µg mL^{-1} FABP) dissolved in PBS what pH at 4°C for overnight. The incubation chambers were blocked with 60 µL per chamber 5% (w/v) PBSTM at 37°C for one hour. The upper covers of the incubation chamber in the vents and microchannel layers are also blocked with PBSTM before finally sealing the disc before testing. The incubation chambers were then washed once using PBS (60 µL per well) and dried using pressurized air. The disc was sealed and taken for testing.

RESULTS

Figures 5 and 6 show the results of the manual benchtop and the LoaD results for the competitive ELISA based detection of spiked FABP and MPO concentrations from 'FABP and MPO-free' human serum, respectively. The LoaD is capable of detecting the CVD biomarker panel from serum within the same range as the benchtop protocol. The sensitivity may be further improved by addressing adverse factors such as denaturation of the proteins on the disc surface and enhanced blocking.

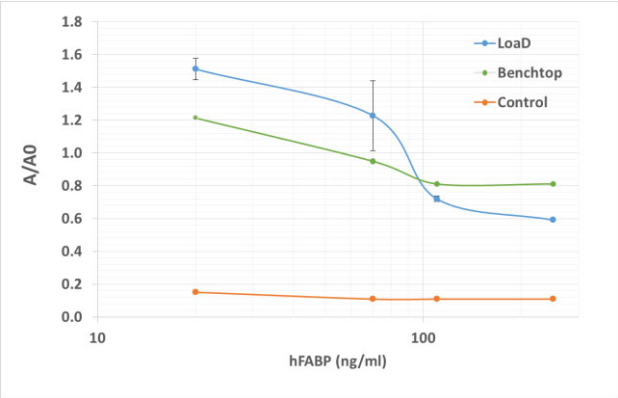

Figure 5: LoaD (blue) versus benchtop (green) competitive ELISA of anti-FABP antibodies in human serum. The A/A0 values were plotted as a function of the target antigen concentration after 10 minutes (The absorbance with no FABP competition was referred to as A0). For the LoaD negative control, the absorbance with 'FABP-free' serum alone was used (n = 2).

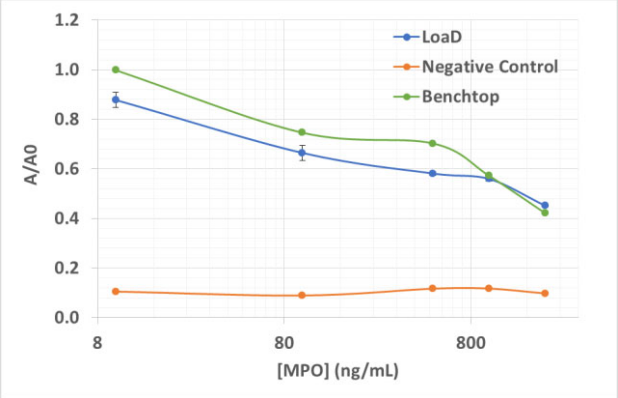

Figure 6: LoaD (blue) versus benchtop (green) competitive ELISA of anti-MPO antibodies in human serum. The A/A0 values were plotted as a function of the target antigen concentration after 10 minutes (The absorbance with no MPO competition was referred to as A0). For the LoaD negative control, the absorbance with purely MPO free serum was used (n = 2).

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CONTACT: *rohit.mishra@dcu.ie*