Microfluidic Particle Separation Techniques for Biomedical Use

András József Laki

Thesis of the PhD Dissertation

Pázmány Péter Catholic University
Faculty of Information Technology and Bionics

Supervisors:
Kristóf Iván, Ph.D.
Pierluigi Civera, Ph.D. †
Danilo Demarchi, Ph.D.

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1 Introduction

Microfluidics concerns design, fabrication, and experiments of miniaturized fluidic systems, which has undergone rapid developments during the last decade [1]. As an interdisciplinary area, this rapidly growing field of technology has numerous applications in biomedical diagnostics, chemical analysis, automotive, and electronics industries [2]. One of the pivotal applications of microfluidics is the development of lab-on-a-chip (LOC) devices as point-of-care (POC) diagnostic tools. A typical LOC device includes various functional modules: sample transportation, sample preparation, separation, detection, and analysis module [3, 4]. The label-free size separation of particles or cells is vital to many of the analytical and preparative techniques used in the fields of chemical, biochemical, and clinical analysis, which led to ground breaking advances in terms of the speed of analyses, the resolution of separations, and the automation of procedures [5]. Additionally, microfluidic separator devices can form a part of portable systems for point-of-care or in-the-field detection [6].

Several variations of microfluidic cell sorters, which implement different sorting mechanisms, have been designed and fabricated [7, 8]. The chosen method of particle handling is generally based on the nature of the application, which strongly depends on the composition of the sample and the final goal of the analysis also should be under consideration. Several strategies exist for this purpose based on specific cell/particle characteristics including manipulation of particles in fluids or removal of particulate matter from fluids[9]. The particles may act or interact with the analyte, in which case they need to be removed from the sample [10].

The integration of particle separation techniques into lab-on-a-chip devices is advantageous, as described by Pamme [11], that these label-free processes are continuous, the separation can be monitored continuously and the sample components are displaced laterally thus each fraction could be collected independently. Based on the applied forces the fractionation could be tangential or perpendicular to the flow direction and can be realised as batch or continuous loading procedures. In batch separation techniques, the particles follow the same paths but at different rates which appears as fractionation over time only; thus, these procedures require precise injection of a very small amount of sample into the separation channel. At the other case, the applied forces have perpendicular components to the flow direction thus the particles are displaced laterally and become separated in space.

2 Enrichment of Blood-borne Pathogens

My aim in the enrichment of blood-borne pathogen was to create novel microfluidic diagnostic tool, which is faster and more efficient than the previous procedures. We believe that this device is well suited for the task of removing parasites from milliliters of blood in order to observe undiscovered parasitosis, and aid their instantaneous detection.

The most widespread technique is the smear test, which is shown in Fig. 1.A and starts with pipetting serological sample onto a glass slide after the hemolysis, the nematodes are counted. The modified Knott's test, which concentrates nematodes by centrifugation and mark specific species by Giemsa stain and shown in Fig. 1.B. First, the anticoagulant blood sample is dissolved 2% formalin in a conical centrifuge tube. After the 5 minutes centrifugation at 1500 rpm, the sediment is mixed by one drop of methylene blue stain coloring the cuticle of nematodes to distinguish better the different nematode spices. Finally, the number of nematodes is counted as is the previous method. The advantage of the concentration method versus the basic serologic methods is the raised detection limit from a bigger sample volume.

I have designed a microfluidic device, called flow-through nematode filter (FTNF), which is shown in Fig. 1.C. This device uses an integrated filtering technique providing the ability to detect much smaller concentration of nematodes from specimens, determine them more accurately and specifically without any external devices reducing the price of the measurement retaining an similar efficiency. The blood sample is forced through the microfluidic device and the pathogens are trapped within the central structure. After the hemolysis, the number of parasites is counter.

The required filtration range of the designed device for nematode filtration comes from parasitology. The nematodes are ovoviviparous and the evolving unsheathed embryo (microfilariae) live in the bloodstream. The length of these microfilariae is $330 - 380$ μm and their width is $5 - 7$ μm [12]. The presented microfluidic device is well-suited for the filtration of these microfilariae, which can be used in similar medical and veterinarian cases.

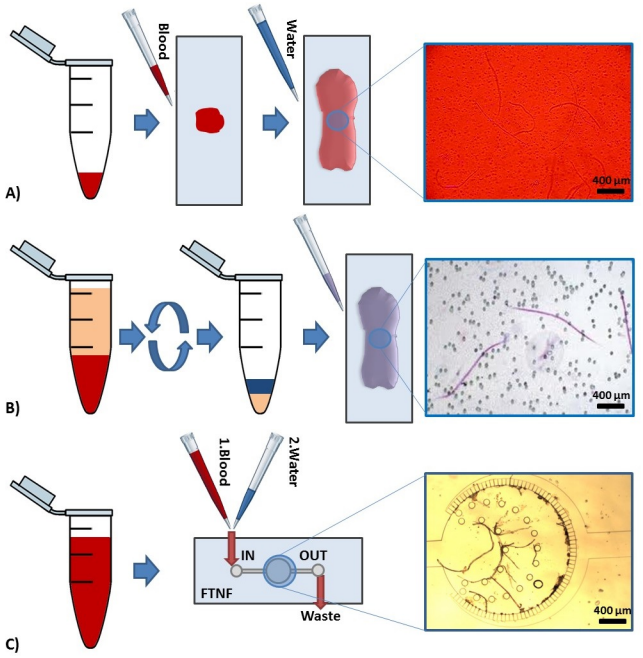

Figure 1: Comparison of detection and diagnostic methods and protocols for dirofilariosis including the proposed simple use FTNF device. A) Blood smear test. Starts with pipetting a drop of serological sample on a glass slide, then hemolyzing with deionized water and finally counting the number of nematodes within the all volume of sample. B) The modified Knott′s test. Anticoagulant blood sample is dissolved 2% formalin in a conical centrifuge tube. After the 5 minutes centrifugation at 1500 rpm, the sediment is mixed by one drop of methylene blue stain coloring the cuticle of nematodes. Finally, the number of nematodes is counted optically. C) Flow-through nematode filter (FTNF). Concentrates the nematodes in the center of the device before the hemolysis from a few ml of blood offering an instantaneous readout.

3 Label-free Separation of Tumor-delivered Extracellular Vesicles

Extracellular vesicles provide a means for cells to interact with each other and appear to play an important role in cancer research and in a

wide variety of pathological processes. The size range of major blood components and the extracellular vesicles is represented in Fig. 2. The separation of the extracellular vesicles takes 6-7 h using several centrifuge steps. The main aim in the separation of tumor-delivered extracellular vesicles was to fractionate submicron particles in a label-free, continuous way. I have chosen a microfluidic separation technique, which does not require an external force.

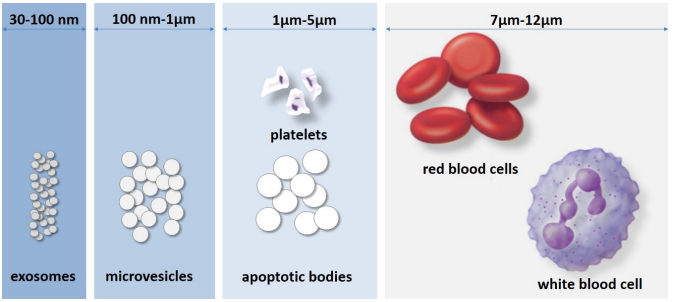

Figure 2: Size ranges of major blood components. While exosomes share size distribution with viruses, microvesicles overlap in size with bacteria, apoptotic bodies and thrombocytes fall into the size range of $1-5\ \mu m$, the diameter of red blood cells is around $6-8\ \mu m$ and the size of lymphocytes is from $7\ \mu m$ up to $12\ \mu m$.

Based on the scientific literature, I have decided to develop a novel microfluidic device to separate tumor-delivered extracellular microvesicles from serological samples. The novelty of the microfluidic device is the continuous and label-free separation of microvesicles across functional laminar streams using an asymmetric micropost array. The applied method was reported first by Huang et al. [13], known as deterministic lateral displacement (DLD). The DLD technique is a size-based particle fractionation procedure which has shown an extremely high size selectivity, adaptability to sorting multiple particle sizes, and a broad range of operating conditions, sorting particles from sub-micrometer scale up to millimeter scale. The designed device has three inlets and a few millimeters long microfluidic channel with this special asymmetric post array.

The DLD array can be considered as a powerful tool for particle separation and manipulation. We can show the evidence that label-free fractionation of micron-scale particles can be delivered by using a DLD array. This suggests that our DLD device is able to provide rapid

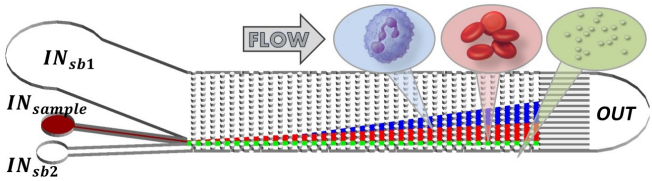

Figure 3: Overview image from the deterministic lateral displacement device. The serological sample (IN_{sample}) is focused by the lateral sheath buffer solutions (IN_{SB1} and IN_{SB1}). The different-sized particles are fractionated along the column structure (leukocytes (blue), erythrocytes (red), and microvesicles (green)).

diagnostic information about the condition of blood cells. The DLD structure can be applied to explore cell-to-cell communication and to fractionate blood sample efficiently for clinical tests without the use of an activation specific label or marker.

4 Research Methodology

In order to achieve the results, the following research methodology has been used:

I have implemented two microfluidic filtration techniques to solve biomedical purposes. A flow-through device has been developed to enrich circulating blood-borne parasites. In the other hand, a continuous label-free separation method, as called deterministic lateral displacement effect, was adapted to separate submicron particles for biological liquids.

In order to design and fabricate the microfluidic devices, computational fluid dynamics simulations were applied to calculate the velocity and pressure profiles of the developed microfluidic structure by COMSOL Multiphysics.

A set of microfluidic channel layouts have been designed by AutoCAD for both cases. 12 similar pathogen filter structures with different capillary width, and a multi-modal array structure have been designed. The microfluidic devices have been fabricated using a standard microfabrication soft-lithographic techniques [14, 15]. The designed microfluidic channels have been lithographed into SU-8 photoresist. Onto the surface of the molds polydimethylsiloxane has been polymerized. The rigid polymer has been pulled off from the mold and after a plasma treatment the polymer has been bond to a glass slide closing the microfluidic channels.

A measurement platform and procedures were developed to test each microfluidic device. Pressure-driven flow was created for each microfluidic device applying syringe pumps. Imaging was performed on an inverted microscope. Fast camera systems have been applied to automize the visualization and the detection procedure of both devices.

5 New scientific results

Thesis group I: I have designed, developed and characterized a novel flow through filter with isobaric filter region for the separation, enrichment and analysis of blood-borne pathogens.

Related publications [L1, L4-L10]

I.1: I have designed and developed a novel device structure using biocompatible materials for the filtration of a wide range of micron-size pathogens.

a) I have designed a novel microcapillary structure arranged in a circular geometry for hydrophoretic filtration of micron-size pathogens with isobaric filtration conditions which us called a flow-through nematode filter(FTNF). I have designed structures with different capillary widths ($W_{capillary}$) from 6.1 μm up to 15.4 μm to test a wide range of pressure and flow conditions for optimization purposes.

b) I have determined the velocity and pressure profile of each FTNF for different flow rates using computational fluid dynamics (CFD) simulations. I have calculated the pressure drop and the flow resistivity of each FTNF structure to develop an isobaric condition in the center of the structure, and to avoid leakages during the experiments. Numerical calculations showed that the designed structures will be able to withstand filtration pressure differences of 1 bar. Also, I have investigated the effect of clogging on pressure drop on the device and found that 50% clogging increases the pressure drop beyond 1 bar.

I.2: I have demonstrated the working principle of the designed nematode filter furthermore, I have determined and measured the efficiency of the FTNF devices and the inhomogeneity of the sample.

a) I have fabricated the FTNF structures with different capillary widths by soft-lithography. I have developed a filtration platform and a 4-step procedure to be used width the FTNF devices.

b) I have determined the efficiency of the FTNF devices with different capillary widths and the inhomogeneity of the blood samples due to particle sedimentation have also been considered during the testing and evaluation. The filtration efficiency was necessary to be introduced due to the implemented filtration method which does not provide 100% percent retention of filtrate. Based on the geometrical parameters of the

device, the filtration settings and the nematode size to be filtered, an average device efficiency parameter could be established for future reference. Due to the sedimentation of the heavier particles (nematodes), inhomogeneity (IH) of the serological sample can occur at low flow rates, which can cause false prediction of the nematode population in the original sample because more concentrated samples filter differently through the device.

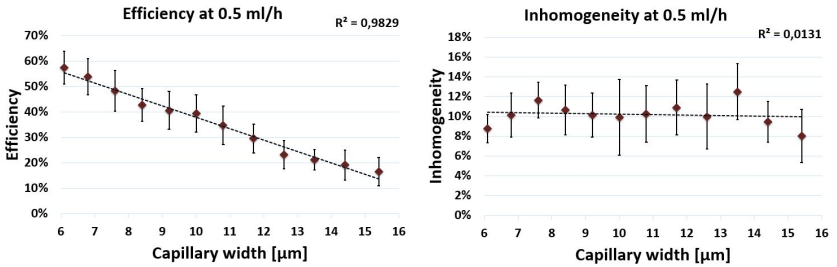

Figure 4: The efficiency and the inhomogeneity of each microfluidic structure ($W_{capillary}$ from 6.1 μm up to 15.4 μm) at 0.5 ml/h flow rate. The error bars of each histogram shows the standard deviations from the mean values.

Testing the filtration devices with nematode infected canine blood, I experimentally measured the efficiency of each FTNF device taking into consideration the inhomogeneity based differences in filtration of the blood sample at different flow rates (0.25 ml/h, 0.5 ml/h and 1 ml/h). I found that the optimal setup was to use 6.1 μm wide capillary structure at 0.5 ml/h flow rate (Fig. 4).

I was able to represent the influence of the flow rate on the inhomogeneity of the sample due to the sedimentation. Based on the measurements, I found that increasing flow rate, the homogeneity of the sample increased.

Decreasing capillary width ($W_{capillary}$), the filtration efficiency increases but beyond a higher volumetric rate the nematodes can be forced through the capillary structure due to the raised pressure drop.

Thesis group II: I have realized a continuous label-free separation of tumor-delivered extracellular vesicles from serological samples by adapting and fine tuning the deterministic lateral displacement (DLD) method. In this novel application area of the method I designed, fabricated and tested separation devices and showed their separation efficiency. I have also studied and extended the physical description of the DLD effect on particles with an inertia-based theory.

Related publications [L2, L10-L15]

II.1: I have developed a multi-modal deterministic lateral displacement array to separate continuously the tumor-delivered extracellular microvesicles from serological samples.

a) I have designed an asymmetric array of cylindrical obstacles implementing the multi-modal deterministic lateral displacement theory. I have calculated the desired critical diameters of each DLD array sections. Each DLD section was designed with cylindric pillars of 20 μm diameter (D_{post}), the gap between adjacent pillars in each columnline (g) is 10 μm, the vertical array period (λ) is 30 μm and the horizontal array period (γ) is 40 μm. The column shift ratio (ϵ_n) which ranges from 0.1 up to 0.33 with steps of 1/60, describes 15 column sections (n) following each other thus the $D_{c,n}$ is between 3.9 μm and 7.7 μm.

b) I have calculated the pressure drop and the flow resistivity of different-height devices to obtain an acceptable channel height and length for the adopted purpose.

c) I have fabricated DLD devices by soft-lithography. I have constructed a microfluidic platform and a procedure to test the DLD devices.

d) I have extended the semi-automated experimental setup with a real-time image processing and particle counting application which required a CNN-based algorithm development to count the number of particles in the final channel section area. I could count the number of cells with this algorithm using an EyeRIS v1.3 camera.

II.2: I have proved experimentally and measured the displacement of the white blood cells, red blood cells, and microvesicles using the DLD structure. Based on the experiments I have created a novel description of the particle migration along the DLD structure.

a) I proved that the proposed label-free fractionation of microvesicle

from blood cells in serological samples can be delivered in practice by using the deterministic lateral displacement array at 1 mm/s flow velocity within a 20 μm high DLD structure with $g = 10$ μm, $\lambda = 30$ μm, and $\Delta\lambda$ varies from 3 up to 10 μm with a step of 0.5 μm. I have measured the displacement of these blood components from the initial position at the final detection area.

b) I have created a novel description of the particle migration along the DLD structure, which considers also the physical parameters of the particles (mass, diameter, and velocity).

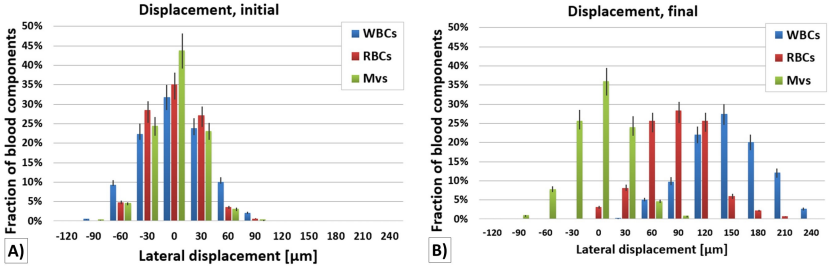

Figure 5: The efficiency of the cell separation using our developed DLD device (white blood cells (WBCs, blue), red blood cells (RBCs, red), and microvesicles (MVs, green)). A) The dispersion of the cell components in the initial section. B) The lateral displacement of the cell components in the final section. The error bar displays the standard deviations.

6 Application of the results

I have developed a flow-through nematode filter (FTNF) to enrich circulating nematodes from native blood, and a deterministic lateral displacement device to separate tumor-derived extracellular vesicles from serological samples.

The developed microfluidic devices can be used as a diagnostic tools for several biomedical purposes as sample preparation, chemical analysis or other industrial applications. Modifying the geometries of both devices, the developed microfluidic structures can be adapted for novel clinical, veterinarian, and industrial cases.

In biomedical sense, these microfluidic devices can replace analytic procedures or tools in clinical applications. Human erythrocytes adopt biconcave disc form. Any change or variety of their shape highlights diseases as sicklemia, infection of malaria, or other blood-borne pathogens.

Another important application can be the observation of the circulating tumor cells (CTCs), which has an important role in cancer metastasis. The clustering of the cancer and the stromal cells could be useful to show the presence of malignant cancers. The continuous observation or filtration of CTCs using the developed microfluidic devices can give us invaluable information.

Further application of the developed microfluidic devices can be the filtration of drinking water. Water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections. The observation and filtration of waterborn pathogens from drinking water using microfluidic devices can be significant in the close future.

7 List of Publications

[L1] **A. J. Laki**, K. Ivan, E. Fok, and P. Civera, *"Filtration of Nematodes using an Integrated Microcapillary System,"* BioNanoSci., pp. 1–11, Oct. 2014.

[L2] **A. J. Laki**, L. Botzheim, K. Ivan, V. Tamasi, and P. Civera, "Separation of Microvesicles from Serological Samples Using Deterministic Lateral Displacement Effect," BioNanoSci., pp. 1–7, Nov. 2014.

[L3] I. N. Huszar, Z. Martonfalvi, **A. J. Laki**, K. Ivan, and M. Kellermayer, "Exclusion-Zone Dynamics Explored with Microfluidics and Optical Tweezers," Entropy, vol. 16, no. 8, pp. 4322–4337, Aug. 2014.

[L4] **A. J. Laki**, G. Z. Nagy, K. Ivan, P. Furjes, O. Jacso, E. Fok, and P. Civera, *"Integrated microcapillary system for microfluidic parasite analysis,"* in 2013 IEEE Biomedical Circuits and Systems Conference (BioCAS), 2013, pp. 118–121.

[L5] **A. J. Laki**, K. Ivan, Z. Fekete, D. Demarchi, and P. Civera, *"Filtration of intravenous cardiopulmonary parasitic nematodes using a cross-flow microfluidic separator,"* presented at the NanoBio-Europe (NBE), 2012.

[L6] **A. J. Laki**, K. Ivan, Z. Fekete, P. Furjes, and P. Civera, *"Filtration of intravenous cardiopulmonary parasitic nematodes using a cross-flow microfluidic separator,"* presented at the EMBL Microfluidics, 2012.

[L7] **A. J. Laki**, K. Ivan, P. Furjes, and P. Civera, *"Integrated microcapillary system for microfluidic parasite analysis,"* presented at the Advances in Microfluidics & Nanofluidics (AMN), 2013.

[L8] **A. J. Laki**, G. Z. Nagy, K. Ivan, P. Furjes, and P. Civera, *"Stand-alone integrated microfluidic parasite analysis system,"* presented at the From Medicine to Bionics, 2013.

[L9] **A. J. Laki**, G. Nagy, K. Ivan, P. Furjes, and P. Civera, *"Stand-alone integrated microfluidic parasite analysis system,"* presented at the NanoBioEurope (NBE), 2013.

[L10] **A. J. Laki**, G. Nagy, K. Ivan, P. Furjes, and P. Civera, *"Stand-alone integrated microfluidic parasite analysis system,"* presented at the International Conference on Biomedical Engineering (ICBME), 2013.

[L11] **A. J. Laki**, L. Botzheim, K. Ivan, T. G. Szabo, V. Tamasi, E. Buzas, and P. Civera, *"Microvesicle Fractionation Using Deterministic Lateral Displacement Effect,"* presented at the IEEE Nano/Micro Engineered and Molecular Systems (IEEE-NEMS), 2014.

[L12] **A. J. Laki**, L. Botzheim, K. Ivan, T. Szabo, E. I. Buzas, and P. Civera, *"Label-Free Fractionation of Tumor-Derived Extracellular*

Vesicles from Human Blood Using Deterministic Lateral Displacement Effect," presented at the Miniaturized Systems for Chemistry and Life Sciences (MicroTAS 2014), 2014.

[L13] **A. J. Laki**, I. Rattalino, A. Sanginario, N. Piacentini, K. Ivan, D. Lapadatu, J. Taylor, D. Demarchi, and P. Civera, *"An integrated and mixed technology LOC hydrodynamic focuser for cell counting application,"* presented at the IEEE Biomedical Circuits and Systems Conference (BioCAS), 2010, pp. 74–77.

[L14] **A. J. Laki**, I. Rattalino, F. Corinto, K. Ivan, D. Demarchi, and P. Civera, *"An integrated LOC hydrodynamic focuser with a CNN-based camera system for cell counting application,"* presented at the IEEE Biomedical Circuits and Systems Conference (BioCAS), 2011, pp. 301–304.

[L15] **A. J. Laki**, A. Sanginario, D. Demarchi, K. Ivan, and P. Civera, *"An Integrated and Mixed Technology LOC Hydrodynamic Focuser for Cell Counting Structures,"* presented at the NanoBio-Europe (NBE), 2011.

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