Final report on NKFIH-OTKA project K108366

"Creating microchannels by Proton Beam Writing and their applications in Lab-on-a-chip devices"

Detailed report

We have achieved the main goal of the project: developed a microfluidic device that is applicable to filter circulating tumor cells (CTCs) out of blood. The summary of our results was published in two parts in the Electrophoresis journal, the first part describing the design and feasibility [1], while the second part was about the image sequence analysis based evaluation and biological application [2].

In the first year we have established our network. We have been performing our tasks at the three participating locations: Atomki, Debrecen; MTA EK MFA, Budapest; Pannon University, Veszprém. The design of the microfluidic devices was performed based on shared ideas, through videoconferences or participating researchers also visited personally others' facilities.

For the design we used computational fluid dynamics (CFD) simulations, which speeded up the prototyping and reduced the cost of development. The preliminary microfluidic separation structures were designed after detailed Finite Element Modelling (FEM) of the hydrodynamic behaviour of the proposed structures. For simulation flow fields and particle distributions the advanced module of the COMSOL Multiphysics were implemented. See an example on Fig. 1.

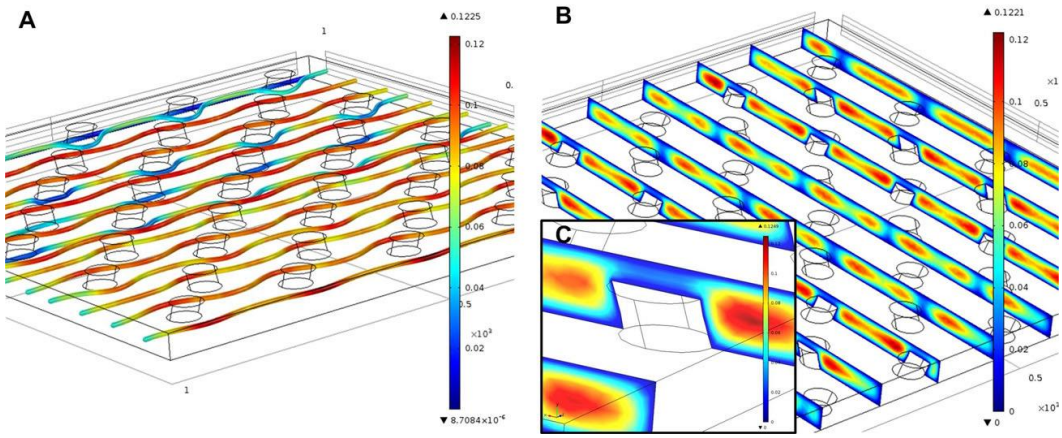

Figure 1. Calculated velocity field distributions inside the functional part of the microfabricated cell capture device. (A) Streamlines with uniform density trace trajectories; (B) slice cuts, perpendicular to the flow direction, representing the velocity distribution to allow detailed investigation of the simulated flow field; (C) magnified view of velocity distribution around a given pillar. Colors indicate the velocity magnitude.

Due to the chemical, biological and technological advantages and achieving optimal compatibility with the different fabrication methods (e.g. UV lithography, Proton Beam Writing (PBW)) we decided to shift the fabrication technology from the silicon based micromachining to the polymer based microfabrication. In our approach the main integrating microfluidic channels were formed by PDMS soft lithography. PDMS is a silicon based organic polymer, which is convenient to realize microfluidic test structures due to its transparency, flexibility, reliable geometry transfer, affordable price and biocompatibility. It is well suited for large scale production and also for fast prototyping. Microfabrication in PDMS is composed of moulding and polymerising on the SU-8 photoresist replica, then removal by peeling off from the moulding form. This conventional method was used in the UV lithography process. The tiny micropillars were formed by PBW also in PDMS, this makes an advantage that the whole chip inside is made of the same material (top, bottom, sides). PBW allows fabricating inclined (tilted) structures which can increase the free surfaces of the microfluidic separation

substructures (micropillars). The methodology of PBW on PDMS was described in detail in the publications [3,4,5,6].

We have tested the feasibility of tilting the samples to different angles before each irradiation step, thus we were able to produce tilted micropillar arrays. To eliminate the secondary charging effects of the glass substrate the MTA EK MFA fabricated special substrates coated with conductive transparent ZnO layer deposited by Atomic Layer Deposition (ALD). We have produced several patterns by PBW into PDMS resist material in liquid phase. The first PDMS layer was spin coated on glass. This was cross-linked by thermal curing. The second PDMS layer was irradiated by proton microbeam in its liquid phase. The irradiated areas were cross-linked, while the un-irradiated areas remained liquid. The process schematic is shown on Fig. 2.

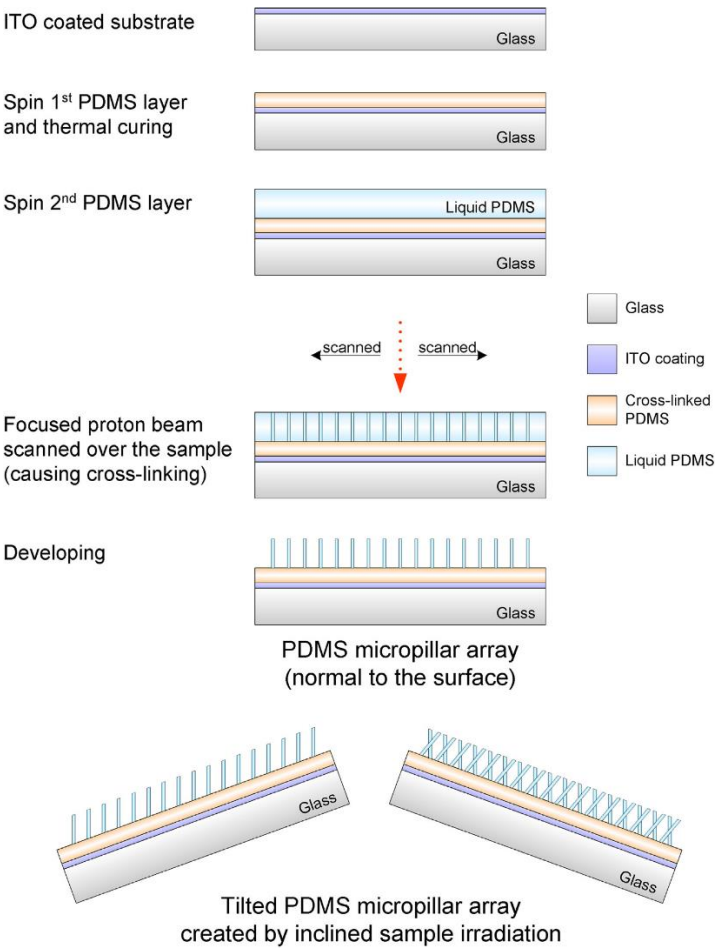

Figure 2. Schematic illustration of the PBW process (side view). The proton beam was magnetically scanned over the resist at an area of 2 mm × 2 mm. The tilted structures were created by turning the sample holder relative to the beam axis.

The final pattern, that was resulted by the numerical simulations, is shown on Fig. 3. For the irradiations we used 2 MeV protons focussed down to 1 μm spot size. The PDMS layer thickness was chosen to be thinner than the range of protons in this material, thus at the end of range the ions reached the substrate, while in the PDMS layer the deposited energy was nearly homogeneous along the depth. We used three different angle settings for the left and right tilted pillars and the alignment crosses. These shown by red, blue and black on left panel of the figure.

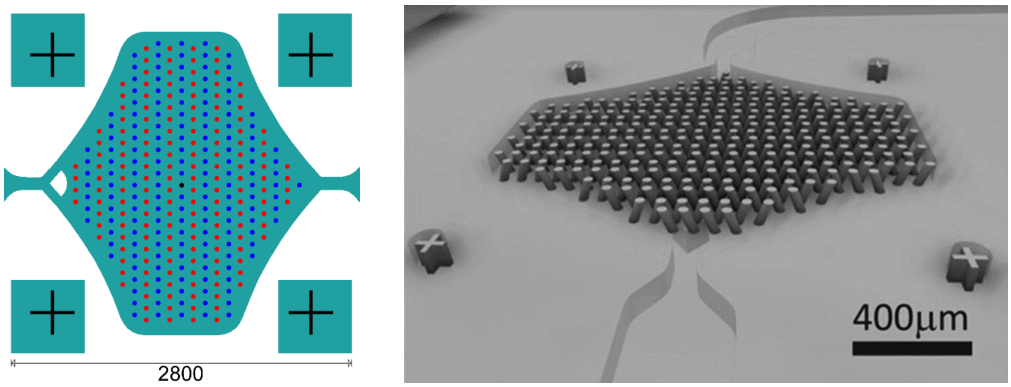

Figure 3. Design of the microfluidic cell capture device with doubly tilted pillar array. Left panel: Bottom part of the chip, patterned by proton beam writing. Red circles were irradiated under +20° tilt angle, while blue circles were irradiated under –20° tilt angle. The pillars were compressed $cos(20°) \approx 0.94$ times in the horizontal direction (rotational) in order to obtain circular-shaped pillars. The alignment marks (black crosses) were irradiated without tilting. Right panel: 3D drawing of the proposed pillar array and the alignment marks for the top and bottom structures to assure proper bonding.

The results of the PBW made tilted micropillar arrays are shown on Fig. 4. We produced several identical microstructures to be able to provide enough central pieces for the assembly of the complete device. It is clear from the SEM photos that the PBW process is very well accurate and reproducible.

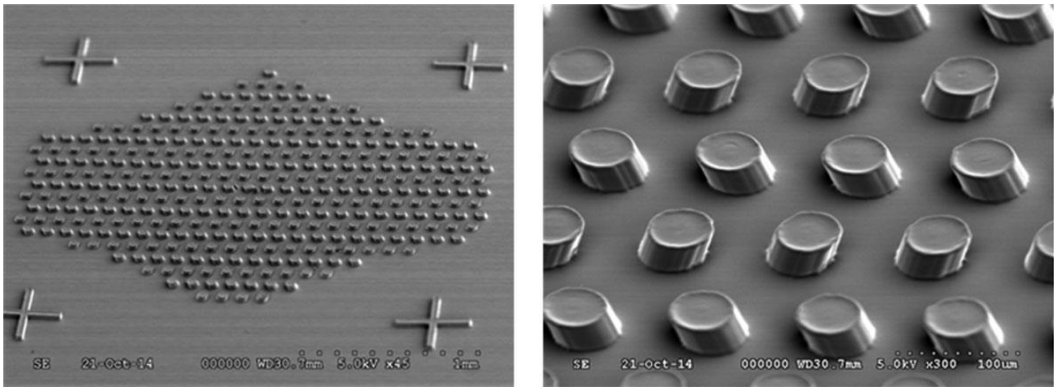

Figure 4. Scanning electron microscopy image of doubly tilted micropillars, fabricated of PDMS by polymerization with focused proton beam on the top of a cross-linked PDMS layer. The right panel shows the magnified view of the pillars.

Sample injecting microfluidic systems were created also in PDMS by soft lithography technique (see Fig. 5) at the MEMS LAB of the MTA EK MFA. SU-8 epoxy based photosensitive polymer was applied as replica for the micromoulding process. The microfluidic chambers were designed to be geometrically compatible with the pillar system and to provide precise connectivity and sample injection during the experiments. Subsequently, the microfabiracated tilted pillar array and the top lid microfluidic system were bonded together using oxygen plasma surface activation.

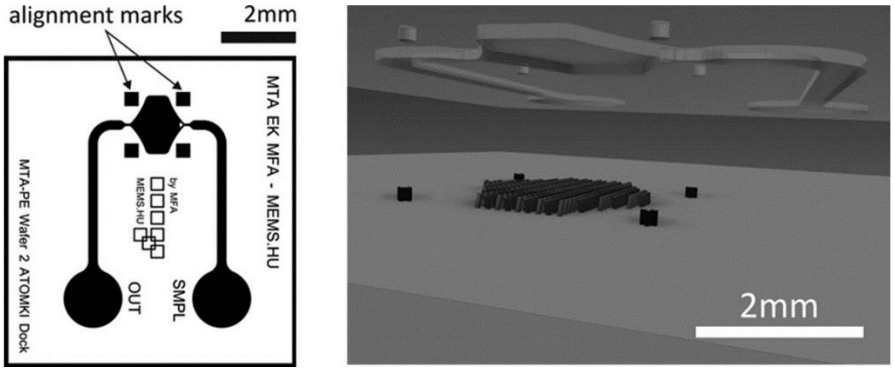

Figure 5. Sample transport microfluidic chip design. Left panel: Chip layout, the dark area (inlet and outlet reservoirs, the channels connecting to the central part of the device, the alignment marks) was exposed to UV light in SU-8 for patterning as molding replica for PDMS casting. Right panel: Alignment schematics. It is visible that the PBW-made bottom part was prepared first, then the UV lithograpy bottom part, and finally the bonding of top and bottom to each other.

The assembled microfluidic device is shown on the photos of Fig. 6. This part of the project was done in Veszprém, where the necessary accessories (e.g. fluid pumps, inverted flurescent microscope, etc.) and the end-user know-how were available. Cell trajectories were recorded using a digital camera microscope objective assembly, which was placed above the flow through microchip device. The recorded image sequences were then analyzed by an in-house developed routine, performed in MATLAB 2015b environment utilizing functions form its Image Processing Toolbox. The motion analysis was based on direct comparison of the actual images against the reference images, i.e., the background. Recorded RGB images were converted to grayscale to reduce their complexity to help the cell tracking algorithm.

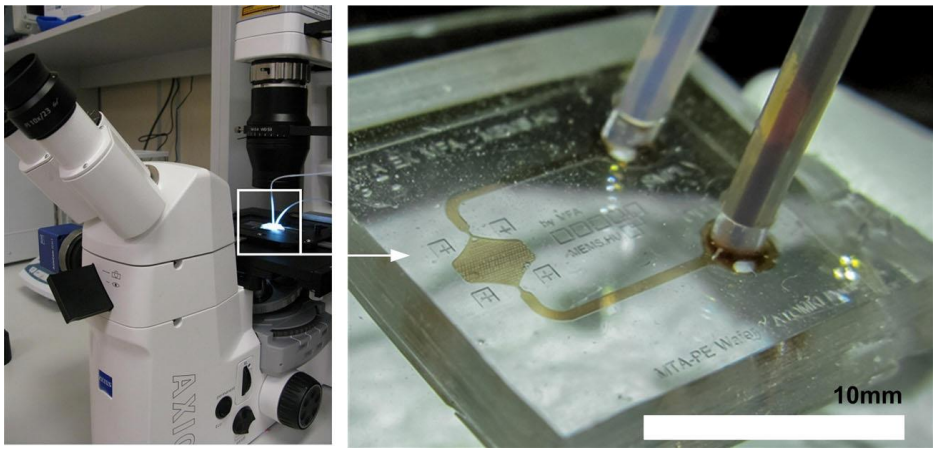

Figure 6. The combined cell capture device. The microfluidic and sorting subsystems were sealed by O_2 plasma enhanced bonding. The image shows the combined capture device filled by a colored biological test solution containing yeast cell culture.

A novel tracking approach (Fig. 7) has been developed in order to validate simulation results with actual experiments. The in-house developed algorithm automatically detects the current position of the flowing particles (tracking agent) inside the prototyped microfluidic cell capture device and calculates the corresponding trajectories. Besides conventional tracking, the developed script also provides dynamic features, which are valuable information about further development possibilities. While rare cells and tumor cells are hard to identify using optical detection system in the range of visible light without phase contrast, thus new biological model system has been composed. Saccharomyces cerevisiae cells have comparable cell size with targeted cells (rare cells and / or tumor cells) while yeast's cell membrane is rather rigid and visible due to relative high chitin content.

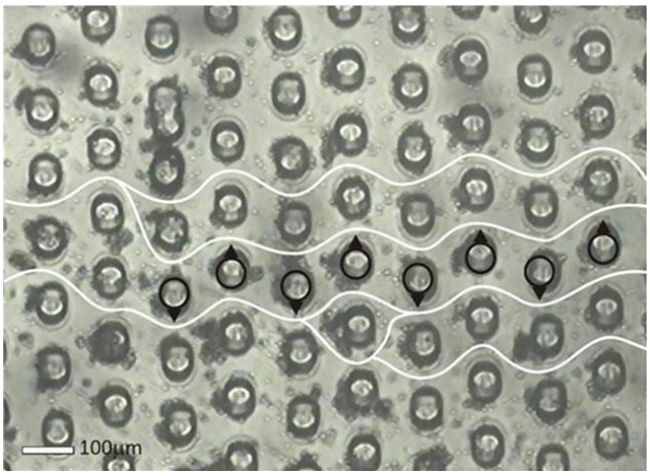

Figure 7. Cell trajectories in the tilted pillar array demonstrating the significant effect of the asymmetric local hydrodynamic parameters evolving around the tilted pillars. The asymmetric pressure and shear force distribution near by the tilted pillars may shift the cells toward the inclined side of the microstructure.

Furthermore, the developed microfluidic cell capture device was tested using real tumor cell lines. The microfluidic systems were surface treated and functionalized by adequate antibody molecules to be applicable for capturing the targeted cell lines. It can be concluded that high efficiency capture of melanoma and colon cells were obtained in the microfluidic device using EpCAM, CD44 and CD66e antibodies immobilized on the improved surface of the tilted micropillars. Cell capture efficiency significantly increased with proper flow characteristics geometry optimized in this study.

The first step of the detection procedure was the edge identification, where the Prewitt edge detection algorithm was applied with 0.02-0.08 threshold. The applied optimal threshold value mainly depended on the image contrast, thus it was automatically adjusted for every image. Comparison of the actual and background images resulted in coordinates of circle shape blobs (built up from at least 50 pixels), which were collected as the actual positions of the cells in the microchip. A moving window approach was used to define the reference image, i.e., the background was handled as the average of 100 previous frames before the actual one. Utilization of the moving window technique was necessary since every successful cell capture event changed the background. The coordinates of each blob in every image and the detection time were collected during the analysis.

Reconstruction of the cell trajectories was based on the calculation of maximal cell movement using the coordinates of each observed cell. Negative differences (cells moving backwards) in coordinates were neglected. The cell identity criteria was set up as 40 pixels, meaning if the difference between two detected cells was less than the criteria then the cell was considered the same that was observed in the previous time step. The accepted coordinates were then acquired and the resulted trajectories evaluated (Fig. 8).

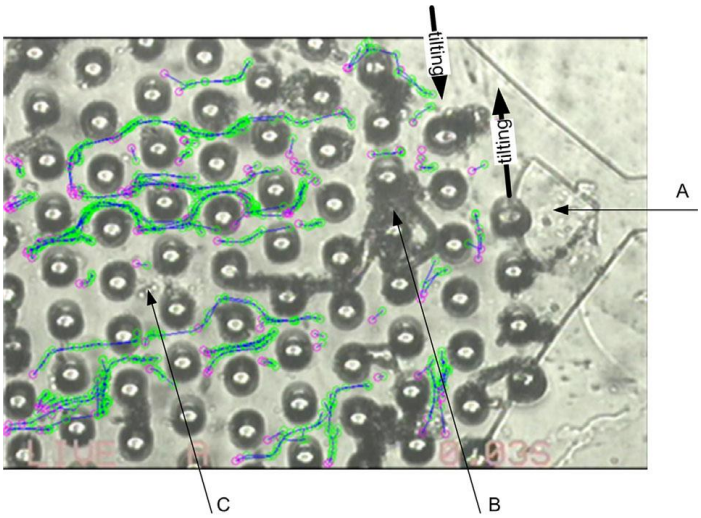

Figure 8. A representative screenshot from the acquired and processed image sequence video, showing the flow distributor (A), a cell cluster (B), which were clogged in the pillar array, and a free flowing cell cluster (C). Blue lines represent the observed cell trajectories reconstructed from the previous positions of the observed cells (green circles), while purple circles denote the ends of the tracked cell pathways.

There are various liquid materials whose elemental composition is of interest in various fields of science and technology. In many cases, sample preparation or the extraction can be complicated, or it would destroy the original environment before the analysis (for example, in the case of biological samples). However, multielement direct analysis of liquid samples can be realized by an external PIXE−PIGE measurement system. Particle-induced X-ray and gamma-ray emission spectroscopy (PIXE, PIGE) techniques were applied in external (in-air) microbeam configuration for the trace and main element determination of liquid samples. The direct analysis of standard solutions of several metal salts and human blood samples (whole blood, blood serum, blood plasma, and formed elements) was realized. From the blood samples, Na, P, S, Cl, K, Ca, Fe, Cu, Zn, and Br elemental concentrations were determined. The focused and scanned ion beam creates an opportunity to analyze very small volume samples (~10 μL). As the sample matrix consists of light elements, the analysis is possible at ppm level. Using this external beam setup, it was found that it is possible to determine elemental composition of small-volume liquid samples routinely, while the liquid samples do not require any preparation processes, and thus, they can be analyzed directly. In the case of lower concentrations, the method is also suitable for the analysis (down to even ~1 ppm level) but with less accuracy and longer measurement times. The experimental setup can be seen of Fig. 9. This result was published in Analytical Chemistry [7].

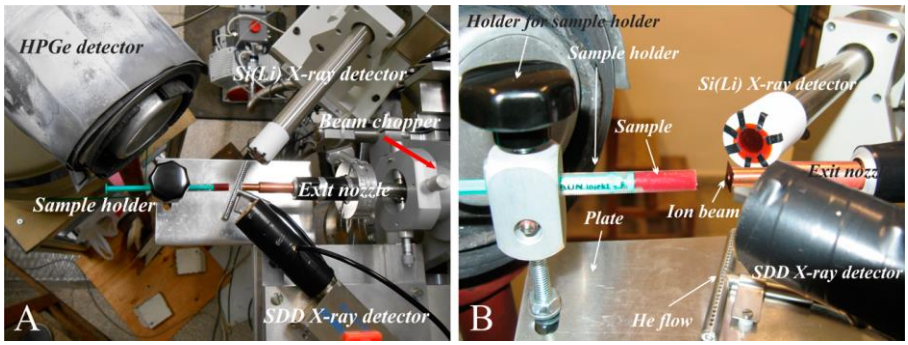

Figure 9. External microbeam setup for the analysis of liquid samples showing the detectors, sample holder and the exit nozzle (A, B).

As an additional task, we have used the PBW method to demonstrate the ability of writing optical waveguides in various materials, which can be integrated not only into optical devices, but also into sensors such as biological applications. A great number of optical crystals and glasses were identified and are used as good optoelectronic materials. However, fabrication of waveguides in some of those materials remains still a challenging task due to their susceptibility to mechanical or chemical damages during processing. PBW was used for direct writing of the channel waveguides in the tellurite glass using focussed beams of 6–11 MeV C3+ and C5+ and 5 MeV N3+. The waveguides were studied by phase contrast and interference microscopy and micro Raman spectroscopy. Guiding properties were demonstrated by the end fire method [8,9].

These irradiations took place on the 3 MV Tandetron accelerator and the scanning nuclear microprobe at the Nuclear Physics Institute, Řež, Czech Republic. These irradiations became feasible after complete realignment (by our coordination) of the microbeam line in Řež, and heavy ion microbeams were demonsrated using the Cs sputter ionsource of the tandem machine.

It is important to emphasize that the required infrastructure for PBW includes a nuclear accelerator, which was in our case the 5 MV Van de Graaff machine of MTA Atomki. The scanning proton microprobe was installed on this accelerator in 1996 by the PI of this project (and this was his PhD topic). Recently Atomki has installed a new state-of-the-art 2 MV Tandetron accelerator manufactured by High Voltage Engineering Europa B.V. which was funded by the MTA Infrastructure grants, and a major upgrade further extended the capabilities (ion sources, beam intensity, beam brightness) by the GINOP-2.3.3-15-2016-00005 project. We have performed the energy calibration of this new machine [10,12]. In order to further develop the focusing performance of the microprobe setup, we conducted ion optics calculations [11], and demonstrated 200 nm beam spot size on the new nanoprobe [13].

Publications

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