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Multiplex determination of antigen specific antibodies with cell binding capability in a self-driven microfluidic system

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ABSTRACT

Antibodies, induced during infections or in autoimmune diseases, exert their protective or destructive effects as part of an orchestrated system that contains several other immune system related proteins and cells. Accordingly immunological assessment of the humoral immune response should include not only the determination of antigen specific antibody level but also the capability of binding to effector cells. We designed and fabricated a polymer based modular autonomous capillary device with an integrated protein microarray that fulfills this need and enables simple, robust detection of antigen specific antibodies through their effector cell binding capability. Serum samples derived from rheumatoid arthritis patients, U937 monocytoid cells and cell stain were sequentially introduced into the self-driven device with disease specific printed citrullinated peptide antigens. The number of bound cells was determined by brightfield microscopic imaging. We found strong positive correlation between antibody level determined by conventional protein microarray technology and binding frequency of U937 cells. Detection by U937 cells was more sensitive than with secondary antibodies for one peptide antigen and less than 13 CV% was experienced in the reproducibility of the measurement.

1. Introduction

Antigen specific antibodies with an important role in protection against infectious diseases are produced after the immune system encounters an antigen. Binding of the generated antibody to antigen may directly neutralize the pathogen, but in most cases solely the binding is not effective enough and further components of the immune system such as the complement system or various effector cells with receptors for the bound antibody (FcR) are required to achieve the protection needed. Attachment of cells to the formed antigen-antibody containing immune complex heavily depends on the molecular properties (isotype, glycosylation) beyond the mere quantity of the antibodies. Accordingly, the selective determination of the concentration of antigen specific antibodies with cell binding properties may provide a biologically relevant readout.

The immune system is a double edged sword however and under autoimmune conditions the produced autoantibodies specific to self-molecules can harm the host organism. Rheumatoid arthritis (RA) is an example of systemic autoimmune diseases that affects 0.5-1% of world population and results in progressive joint destruction in patients [1]. The presence of anti-citrullinated peptide antibodies is a diagnostic marker for RA. In our assay we tested two such citrullinated peptides, namely VCP2 [2] and HCP2 [3] as model antigens targeted by proinflammatory autoantibodies.

Myeloid cells and their progeny, especially monocytes, macrophages, neutrophil granulocytes and dendritic cells are

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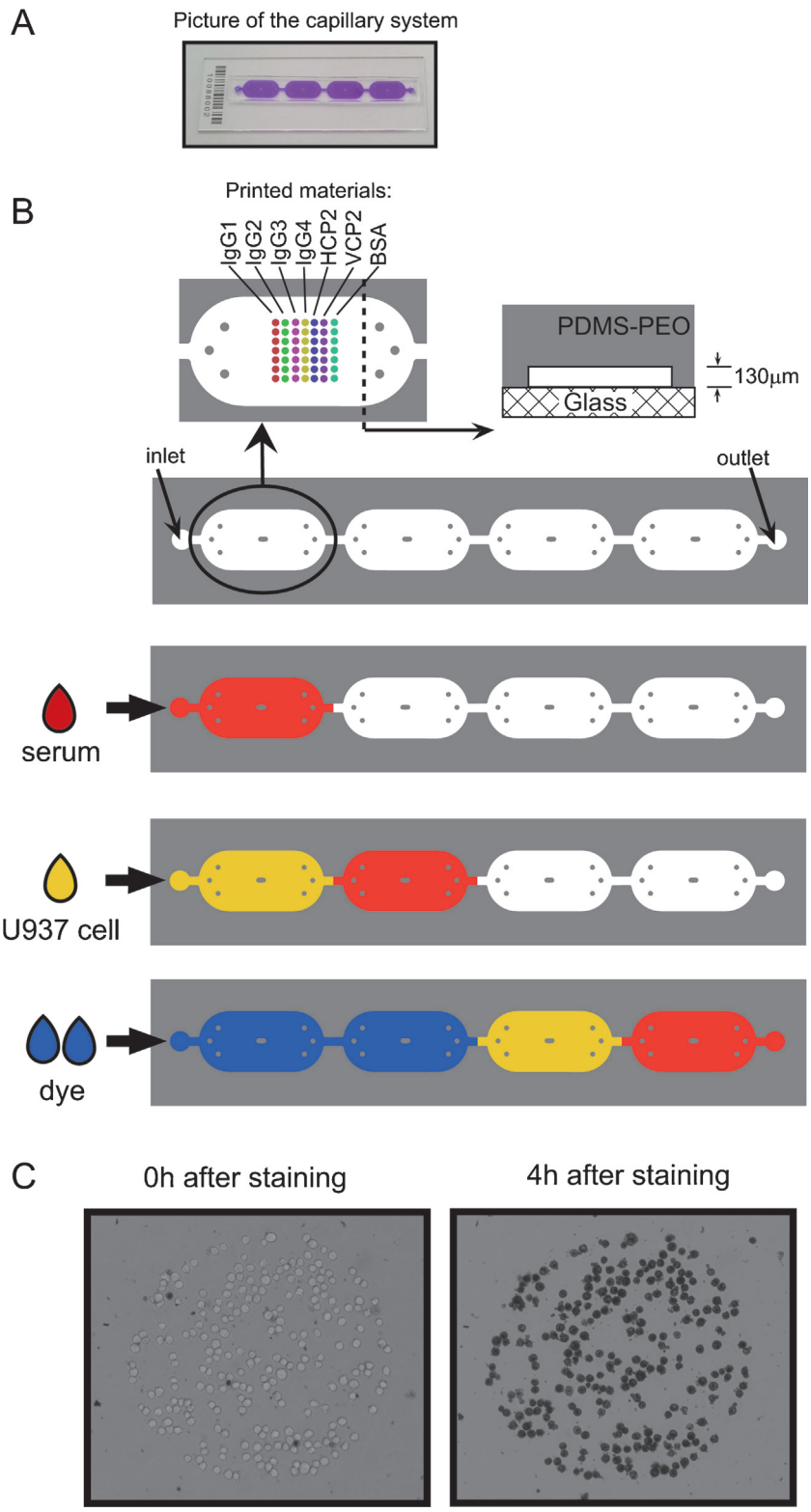

Fig. 1. Layout of experimental design. A) The photo shows the capillary system filled with blue stain for better visualization. B) Tested materials were printed in the marked positions of the first chamber area on hydrogel coated glass slides. Microfluidic device was formed by aligned attachment of PDMS-PEO based flow cell containing microfluidic channels onto the biofunctionalised slide. The four sequentially connected chambers are indicated here with white color. The upper right picture indicates the cross-sectional view of the device. First tested serum sample then U937 cell suspension, and finally methylene blue dye solution was added into the input of the capillary. C) Microscopic picture shows that all the spot bound cells stained and were ready for detection after 4 h incubation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

responsible for binding, processing and removing of the immune complexes. The U937 cell line [4] is widely used for modeling human monocytes and macrophages. U937 cells express two activating FcγRs: the high-affinity FcγRI (CD64) and the low-affinity FcγRIIA (CD32) [5]. FcγRI preferentially binds IgG1 and IgG3 over IgG4 and not IgG2. The FcγRIIA variant R131 expressed on U937

cells (Valenzuela 2015 Am J Transplant) binds IgG1 and IgG3 comparably, and IgG4 with a lower affinity, and binds IgG2 weakly, compared to variant H131 [6]. A recent study confirms that U937 cells do not express the inhibitory FcγRIIB [7].

We have shown that probing immune complexes directly by U937 cells on antigen microarrays enables the comprehensive detection of antigen specific antibodies with cell binding capability [5,8]. These observations highlighted the crucial need for a simple and robust sample handling fluidics platform for these experiments to be reliable and reproducible.

Our aim was to integrate protein microarray technology in a microfluidic system to measure the level of antigen specific antibodies through cell binding properties and to validate the applicability of the developed system. To this end we designed a modular autonomous microfluidic chamber system that would allow the gentle but controlled sequential treatment of a protein array with a test serum, detecting cells and detection reagents. Due to the combination of the special surface modification and the microscale architecture we were able to merge the advantage of the cheap lateral flow assays and the precise sample dosing systems. In contrast to other conventional techniques, we could test several antigens parallel in a single chamber and we could increase reliability by applying internal controls. Here we report the characterization of this system.

2. Materials and methods

2.1. Serum samples and cells

The ethics committee of Hungary (Egészségügyi Tudományos Tanács, Tudományos és Kutatásetikai Bizottság) gave its approval for conducting study with the following contract number: 24973-1/2012/EKU (658/PI/2012.). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki declaration of 1975, as revised in 2008. Written informed consent was obtained from each participant. Serum samples were collected from 14 rheumatoid arthritis (RA) patients. The diagnosis of the RA was established on the basis of the revised ACR/EULAR classification criteria [9].

U937 monocytoid human cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM Na-pyruvate and was maintained in a humidified incubator (5% CO_2, 37 °C). All materials were purchased from Sigma.

2.2. Preparation of capillary system

The molding replica for microfluidic capillary system was fabricated by soft-lithography technique applying SU-8 pattern. Double layer of SU-8 2005 (MicroChem) epoxy based negative photoresist was spin coated onto a silicon wafer using a Brewer Science Cee 200CBX coat-bake system at 4000 rpm, and patterned by UV-lithography. The mask for the UV-lithography was manufactured by a Heidelberg DWL 66+ laser pattern generator. The prebaked SU-8 film was exposed to UV-light in a Süss Mikrotech MA6 mask aligner, then the unexposed resist was removed in 1-methoxy-2-propyl acetate solution. The structured photoresist pattern was baked at 90 °C as post-exposure bake [10].

Sylgard 184 polydimethylsiloxane (PDMS) precursor, cross-linking agent (Dow Corning) and dimethylsiloxane ethylene oxide block copolymer (PDMS-b-PEO) (Sigma) were mixed in 100:10:0.75 (V/V) ratio. The prepolymer was poured over the moulding form and cured for 1 h at 80 °C. The PDMS-PEO polymer was peeled off from the master and holes were punched for inlet and outlet. The modified structural material of the microfluidic system has advantageous hydrophilic characteristics due to the embedded PEO chains which are capable to quickly diffuse in the polymer matrix and to modify the surface properties of the channel walls [11,12].

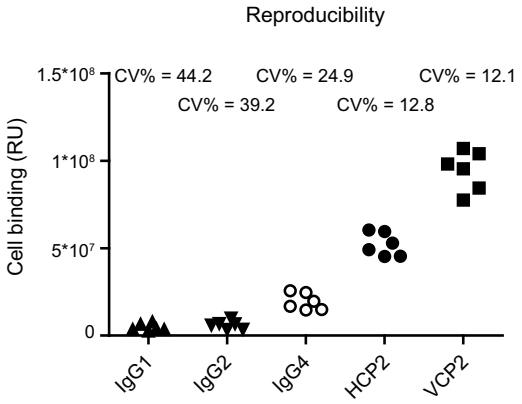

Fig. 2. Reproducibility of cell binding measurement. Graph shows cell binding to IgG subclasses and RA specific peptides following treatment with the same serum in six replicated experiments. Values on IgG3 spots were used for normalization so these data were not plotted. Coefficient of variation (CV%) of reproducibility was calculated. RU: relative unit.

Rheumatoid arthritis disease specific 1 mg/ml HCP2 and 2 mg/ml VCP2 citrullinated peptides (prepared at the University of Florence) were printed in seven parallel spots along with 0.5 mg/ml human IgG1, IgG2, IgG3, IgG4 and bovine serum albumin (BSA) control materials (Sigma) onto Nexterion H hydrogel coated slides by Calligrapher miniarrayer (BioRad). Printed slides were incubated at 37 °C in humidified chamber for 1 h to facilitate the covalent binding reaction and then unbound reactive groups were blocked by incubation in 100 mM Tris for 1 h. Following 3 × 5 min washing in PBS, slides were rinsed in water then dried.

Finally PDMS-PEO microfluidic blocks were simply attached onto printed slides to form capillary sample loading system (Fig. 1.). As only capillary force transfers the solution during the experiment, there is no need for extra bounding process. When the PDMS-PEO block is inserted onto the slides it will properly stick alone. Plasma treatment would result stronger binding, but the procedure would be destructive for the functionalized slides. The geometric parameters of the chambers were defined according to the sample loading protocol and the applied sample volumes.

2.3. Detection of cell binding in capillary system

For reduction of evaporation during the experimental procedure capillary system was incubated in humidified chamber for 1 h before the assay and kept in this chamber during the subsequent incubation steps. Serum samples were diluted 5 times in PBS containing 5% BSA, 2.5 mM $CaCl_2$ and 0.7 mM $MgCl_2$. 10 μl diluted serum was pipetted to the inlet of the microfludic system and after the capillary force filled the first chamber autonomously, it was incubated at 37 °C for 15 min. U937 monocytoid cell suspension was prepared that contained $3 * 10^7$ cell/ml in RPMI medium supplemented with 10% fetal calf serum. 10 μl cell suspension was added to the inlet with pipette and incubated at 37 °C for 30 min. Finally 20 μl of 0.25% methylene blue in PBS was pipetted to the capillary system and after incubation at room temperature for 4 h the bound cells were visualized by Leica DMS1000 microscope (Fig. 1). Diameter of various control spots was around 250 μm, while the peptide spots were larger 400 μm. As the average diameter of U937 cell is 10 μm [13], theoretically maximum 560 cells can bind to the control and 1400 cells to the peptide spots. GenePix

Pro 6.0 software was used to align circular mask to the printed antigen bound cell containing spots. Sum of pixel intensities in the spot minus median of local background multiplied by pixel number in the spot was calculated that reflects the number of bound cells.

2.4. Measurement of peptide specific antibody level

RA specific citrullinated peptide antigens VCP2 and HCP2 were printed in 4 mg/ml, 2 mg/ml, and 1 mg/ml concentration along with 0.5 mg/ml human IgG control in triplicates onto 16-pad nitrocellulose-covered FAST slides (Main Manufacturing) by sciFlexarrayer S11 (Scienion AG, sciArraying service). Dried arrays were rinsed for 15 min in PBS before use, then incubated with 5 times diluted serum in 25 mM EDTA, 0.05% Tween 20 and 5% BSA complemented PBS buffer at 37 °C for 1 h. Serum treated slides were washed with PBS containing 0.05% Tween 20, then incubated in 1:2000 diluted DyLight 649-conjugated F(ab')$_2$ fragment goat anti-human IgG (gamma chain specific) (Jackson ImmunoResearch). Labeling with fluorescent antibodies was carried out at room temperature for 30 min in PBS containing 5% BSA and 0.05% Tween 20. After washing in PBS containing 0.05% Tween 20, arrays were dried and scanned by Axon GenePix 4200A (Molecular Devices) equipped with GenePix Pro 7.0 software. Fluorescent intensity was calculated for each spot as the median fluorescence intensity of the feature, minus fluorescence intensity of the local background. The median of the three parallel spots was normalized to the signals on printed human IgG features for correction of the technical errors.

3. Results and discussion

We have taken into consideration the following factors for the design of our microfluidic device: 1, Materials contacting the sample and reagents should show appropriate hydrophilicity to allow that the system fills up autonomously but with a relatively slow speed to gently detach and remove unbound cells; 2, a modular structure that both admits adequate sample and reagents volumes, which can be precisely pipetted, allows visual control of filling and shows quasi-linear flow properties. The first goal was achieved by using a hybrid system made up of a hydrogel coated microscope slide in combination with PEO-modified PDMS. The structure we identified as optimal was a serially connected row of round-edged chambers with an internal height of 130 micrometers. The self-driven autonomous capillary system designed and fabricated for the measurement of antigen specific antibodies with cell binding capabilities is shown in Fig. 1A.

3.1. Setting up cell binding measurement in the microfluidic device

To test this capillary system, rheumatoid arthritis specific citrullinated peptide antigens (HCP2, VCP2) were printed besides human IgG1 to IgG4 isotypes as positive controls and bovine serum albumin (BSA) as negative control onto 3D thin film coated Nexterion H glass slides (Fig. 1B). The PDMS-PEO based fluidic chip containing specific microstructures was attached onto the biofunctionalised

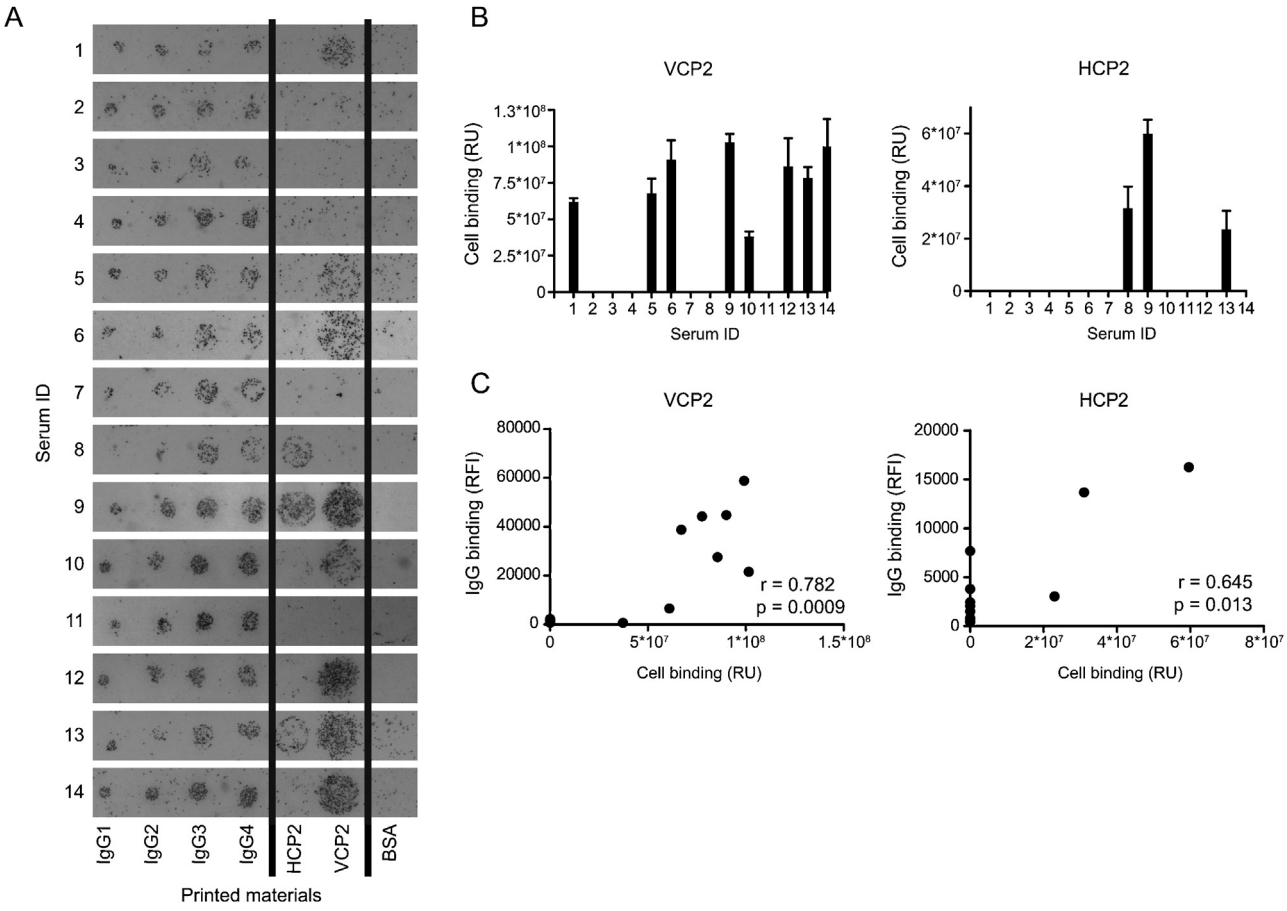

Fig. 3. Detection of rheumatoid arthritis specific autoantibodies with cell binding capability. U937 cells were added following incubation of printed materials with the serum samples. A) Microscopic pictures of spotted antigen bound cells in case of 14 tested serum samples. Only single representative spot row is presented from the 7 parallels. B) Bar graphs show the amount of RA specific peptide (VCP2, HCP2) bound cells following incubation in RA serum samples. C) VCP2 and HCP2 specific IgG levels, determined by protein microarray, were plotted as a function of the amount of bound cells. The same concentrations of peptides were printed for both methods. Spearman r correlation coefficient was calculated and written on the graph. RFI: relative fluorescence intensity.

surface resulting in sealed microfluidic channels with hydrophilic surface characteristics where capillary forces could drive the sample solution along the fluidic pass. Confirming the adequate surface modification we did not need any external devices like centrifuge in a spinning disc device [14] or external pumps [15] for the production of the distractive force: the sample autonomously filled the capillary system and the geometry of the applied structure could control the flow rate. As the tested serum filled the first chamber over the printed area, antibodies bound to the immobilized antigens and immune complexes formed in specific spots. U937 monocytoid cells attached onto the spots containing immune complexes when the chamber was filled with cell suspension in the subsequent step. After incubation, methylene blue dye solution was introduced with the dual function of washing away unbound cells and staining immune complex-attached cells. The applied dye stains only dead cells so we needed 4 h till all cells developed color (Fig. 1C). The attached cells could then be visualized with a conventional light microscope. Since several factors can affect the number of bound cells – like concentration of cell suspension, actual condition of cells or shear force of the washing solution – an internal normalization procedure was applied to compensate for the possible effects of these factors. Printed IgG3 molecules, the most potent activator of U937 cell through Fc receptors were used for normalization. In our system the coefficient of variation was less than 13% during measurement of VCP2 and HCP2 specific antibodies with cell binding capability in case of the series of repeated measurements (Fig. 2.)

3.2. Detection RA specific antibodies with cell binding capability

Serum samples of fifteen rheumatoid arthritis patients were tested in our cell binding assay and the VCP2 and HCP2 peptide specific serum reactivity of these samples were determined based on their ability to trigger cell adhesion compared to hIgG (Fig. 3A). Each serum sample was characterized by the sum of pixel intensities in each antigen feature, indicating the number of adhered cells (Fig. 3B). Antigen specific IgG levels were determined from the same RA serum samples by antigen microarray technique and strong positive correlation was found between the measured antibody levels and the amount of bound cells (Fig. 3C). Detection of VCP2 peptide specific antibody by U937 cell binding was more sensitive than measurement applying secondary antibody. We suggest that for even more patient specific results, purified neutrophil granulocytes from the same patient could be applied, in which case one could also take into account the polymorphic difference of Fc receptors that has substantial effect on binding affinities [6].

4. Conclusions

Here we present a general cell based method that combines antigen microarray with microfluidics and enables the selective detection of antibodies which specifically bind to a chosen antigen and have cell binding capability at the same time. Due to the capillary force transport mechanism of the sample and reagent solutions in this bioanalytical device the test can be carried out easily without any external devices. This feature could enable the construction of low cost, small and autonomous devices for point-of-care cellular diagnostics.

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Biographies

Krisztián Papp is a senior research fellow at the MTA-ELTE Immunology Research Group and member of the Immunomicrotechnology Group. He received the MSc degree in molecular biology/immunology and in 2008 his PhD in immunology from Eötvös Loránd University, Budapest. His main areas of interest are studying the effector functions of antibodies and the development of diagnostic immunological assays using microarray and microfluidics platforms.

Eszter Holczer is a junior research fellow at the Institute of Technical Physics and Materials Science HAS and a PhD student at the Budapest University of Technology and Economics. She received her MSc in Chemical Engineering in 2014 from the Budapest University of Technology and Economics. Her PhD work aims to study and modify the surface properties of the most common substrate materials in microfluidic and MEMS technology to allow the development microfluidic and bioanalytical devices capable to recognize disease specific molecular markers or cells from different samples.

Csilla Kecse-Nagy obtained her B.Sc. and M.Sc. degree in biology from the Eötvös Loránd University in 2012 and 2014, respectively, specializing in immunology. She was working at Synlab Hungary Ltd. Immunology Laboratory in 2014 as lab assistant. She is currently a Ph.D. student at Eötvös Loránd University. Her research interest includes studying the activation processes of neutrophil granulocytes using microfluidic system.

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Veronika Lóránd is currently working as a rheumatology resident at the Polyclinic of the Hospitaller Brothers of St. John of God in Budapest while finishing her PhD work. After graduating from the University of Pécs Medical School in 2012 she worked as a PhD student at the Department of Rheumatology and Immunology of the University at Pécs for three years.

Paolo Rovero graduated in chemistry at the University of Florence in 1983. In 1985–1986 he specialized in peptide chemistry at the University of Sherbrooke, Canada. In 1986 he joined Menarini Pharmaceuticals (Florence, Italy) as Researcher and in 1991 moved to the National Research Council (CNR) in Pisa, Italy. In 1998 he was appointed Associate Professor of medicinal chemistry at the University of Salerno (Italy) and in 2004 moved at the University of Florence (Italy). His research interest focuses on the design and synthesis of synthetic peptides, with special attention to immunological and diagnostic applications, including biosensors.

József Prechl received his PhD in immunology from Eötvös Loránd University, Budapest, and his MD from Semmelweis University, Budapest. Currently he is Director of Research and Development at Diagnosticum zrt, Budapest, Hungary, and leads the Immunomicrotechnology Group of the Office for Research Groups Attached to Universities and Other Institutions of the Hungarian Academy of Sciences, placed at Eötvös Loránd University. His main area of interest is the development of diagnostic immunological assays using microarray and microfluidics platforms.

Péter Fürjes is senior research fellow in MEMS and BioMEMS R&D with the Hungarian Academy of Sciences. He received an MSc in Engineering Physics in 1999, MBA in 2002 and obtained his Ph.D. degree in 2004 from the Budapest University of Technology and Economics. While with the Institute of Technical Physics and Materials Science, Budapest from 2004 he was working in the field of micromechanics and sensor R&D including research assignments with Tateyama Kagaku Ind. Co. (Toyama, Japan) and at Furtwangen University (Furtwangen, Germany). He studied the thermo-mechanical and fluid-dynamical functional behaviour of gas, humidity and flow sensors. His current research is focused at the development and analysis of BioMEMS devices, of micro- and nanofluidic principles for medical applications, including modelling and design along with the development of micro- and nanofabrication processes.