COMBINATION OF DIELECTROPHORESIS AND CENTRIFUGATION FOR CHIP-BASED CELL SEPARATION

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The separation of different cell types in a mixed suspension is an important task in biotechnology and medicine, especially in blood-based diagnostics. For this purpose, a number of chip-sized flow-through systems, so-called "lab-on-chips" (LOCs), were presented by various groups. In many LOCs, the cell manipulation, e. g. sorting, is performed by exerting forces on suspension cells by applying a high-frequency electric field generated by microelectrodes. This technique, dielectrophoresis (DEP) was repeatedly shown to be particularly stress-free for live cells. However, another pre-condition of LOCs yet lacks a satisfying solution, namely the transport of the cell suspension through the microchannels. The method for creating this fluid flow must be at the same time cheap, easy to handle, suitable for different channel dimensions, compatible with requirements of sterility and should not interfere with, or be dependent on, the suspension composition. Here, we report on a DEP-based chip system for the separation of cells in blood samples which has the potential to accomplish these requirements. While the fluid in the channels is at rest, the cell sample is moved through it by centrifugation forces. Simultaneously, a sorting step can be performed. The complete chip set-up is integrated into a CD-shaped disk and rotated on a modified lab bench centrifuge. Our cell manipulation system, therefore, avoids the expensive and clumsy fluidic periphery needed by most current LOCs In the first set of experiments, cell-sized artificial polystyrene particles were moved into either of two branches of a Y-shaped channel. Secondly, a suspension of human lymphocytes and a diluted whole-blood sample were used. In both cases, the particles were aligned and deflected by the DEP forces of a 3D microelectrode arrangement on the top and bottom of the channels. The results indicate the feasibility and the high potential of combining DEP with centrifugation.

Keywords: centrifugation, dielectrophoresis

1. Introduction

Separating cells from a mixed sample is a standard task in biotechnology and modern medicine. E. g., to isolate cells that are of interest for an autologous therapy from a biopsy, a minute percentage of target cells must be separated from a large amount of unwanted cells. Similarly, the separation of those cells that were successfully transfected with a genetic modification in biotechnology should provide a high survivability of the processed cells. Another example is found in transfusion medicine where the proportion of donated blood platelets that has to be discarded for the risk of bacterial contaminations - about one seventh - poses a heavy economic burden in blood banks. Testing of these samples reveals that only one in 2,000 to 3,000 is actually contaminated. Finally, a LOC for separating and enriching food contaminating bacteria was recently described [Yang].

Almost ninety years ago, Westergren used differences in the density of cells to separate blood. Today, centrifugation in vials and magnetic bead techniques are routine procedures. However, they cannot be applied to every sample. They fail if e. g. the sample volume is very small or the cells are highly sensitive to mechanical contact like adult stem cells. Therefore, a range of methods was developed to handle low cell numbers. Unfortunately, only few are gentle enough for the manipulation of delicate cells, dielectrophoresis (DEP) being one. In DEP, suspended particles are exposed to an electric field which polarizes them [Stuke, Pohl]. The induced electric dipole is then repelled from the same electric field. Thus, the particle experiences a translating force. Note, that the particles need not be charged as e. g. in electrophoresis. They only need to be polarisable which is the case for practically all biological material. To avoid electric field effects on the cells, usually radio-frequency electric fields are used because biological systems lack sensors in this frequency range. The use of microelectrodes paved the way for single-cell manipulation with DEP. Common systems utilize DEP elements for sorting particles in microchannels, for washing them by deflection into a parallel fluid flow and back and for characterizing them by contact-freely trapping the objects in DEP field cages [Gascoyne, Müller, Kentsch, Duschl].

All single-cell manipulation relies on methods that transport the cell sample into a microfluidic channel system. Usually, external syringe pumps are employed for this purpose. Their drawbacks are an inherent pulsation and the high, at times prohibitive, costs. On-chip pumping can be induced by electroosmosis and traveling electric waves (TW). While electroosmosis electrodes inevitably cause modifications in the sample due to electrochemical processes, this disadvantage is avoided in TW by the use of high-frequency electric fields [Felten]. However, TW requires a large number of electrodes, usually at least ten. Centrifugation represents a promising alternative to fulfill the outlined demands. It merges the two advantages of handling small samples on a small periphery. Centrifugation was previously employed for the analysis of fluids in a disk-like LOC [Grumann, Brenner]. On it, it is possible to e. g. mix different liquids in a defined manner, initiate a chemical reaction and, finally to analyze the products. The small sample volumes result in short processing times. One proposed application comprises a blood analyzer measuring hematocrit, glucose, ethanol and hemoglobin, another is intended to test for pathogens like tetanus, diphtheria and measles.

Here, we follow a novel approach by fixing the DEP chip to a spinning disk, so that the

centrifugation force moves the suspended particles along the separation channel. We investigate the potential of this set-up by evaluating the separation of artificial polystyrene particles as well as of live human cells. The final aim could be a stand-alone system for the routine separation of cell samples in a lab environment. It would combine the benefits of both DEP and centrifugation yielding a simple and robust method for handling delicate specimens in small sample volumes.

2. Materials and Methods

2.1. *Microfluidic device*

The microsystem consists of two glass slides separated by a 40 µm polymer spacer. One of the two slides is 150 µm thick to provide optimal optical accessibility. Platinum microelectrodes with a thickness of 200 nm are processed photolithographically on the glass slides. The connection leads of these electrodes were passivated where necessary by depositing a 200 nm layer of silicon nitride (Si_3N_4). The glass slides are mounted so as to position the identically shaped microelectrodes on both slides precisely above each other. Thus, a Y-shaped channel with integrated electrodes, one fluidic inlet and two outlets is formed (Fig. 1a). Upstream of the branching point, the channel width is 600 µm. The chip is mounted on a board which provides the connection to the generator and microswitches for control of the different DEP elements.

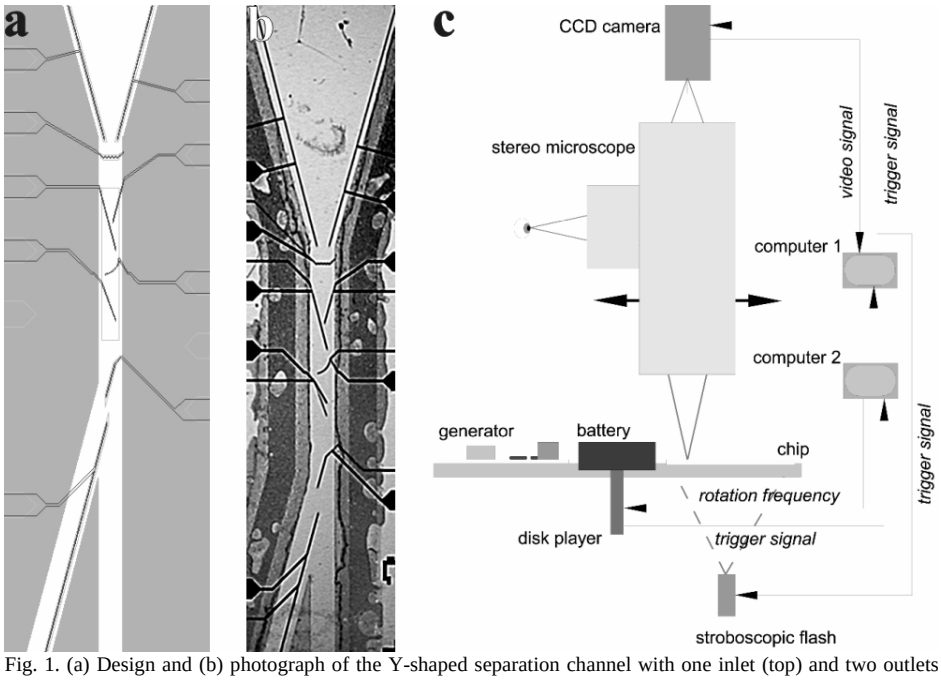

Fig. 1. (a) Design and (b) photograph of the Y-shaped separation channel with one inlet (top) and two outlets (bottom). In this orientation, the axis of rotation is above the images. Before the branching, the channel with is

600 µm. The microelectrodes are processed on both the channel top and bottom and positioned accurately above each other. (c) Scheme of the DEP / centrifugation set-up.

2.2. *Centrifugation set-up*

The microfluidic DEP device is mounted on a PMMA disk together with the generator and two 9 V battery packs (Fig. 2). The disk is spun by a purpose-made electric rotor the speed and direction of which can be computer-controlled. A stereo microscope (Leica MZ12.5, Germany) equipped with a CCD camera (PCO Sensicam, Germany) provides microscopic control. It is fixed to a motorized linear stage, so that its distance from the axes of rotation can be adjusted. To avoid blurring of the image due to the fast moving object, an exposure time of the camera of only 100 ns is used. All microscopic images shown here are filtered and contrast adjusted. The camera and a stroboscopic flash are triggered by a computer according to the rotational frequency. The centrifugal accelerations at 5 Hz, 10 Hz, 15 Hz, 20 Hz, 25 Hz, and 30 Hz are (3.8 ± 0.9) g, (15 ± 4) g, (34 ± 9) g, (60 ± 20) g, (90 ± 20) g and (140 ± 30) g, respectively. The deviations are caused by the different distances of both channel ends from the axis of rotation.

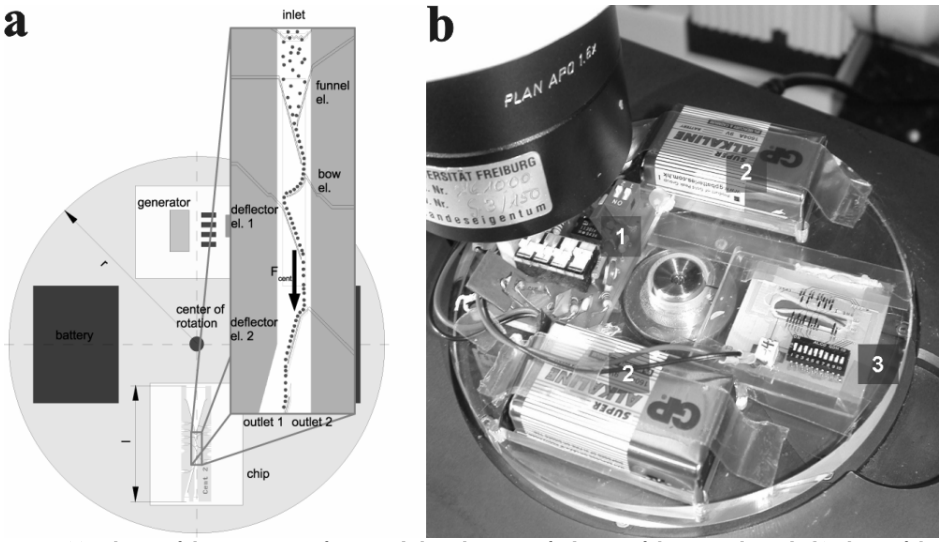

Fig. 2. (a) Scheme of the DEP / centrifugation disk with a magnified view of the microchannel. (b) Photo of the centrifugation disk. All parts are fixed with hot-melt adhesive and tape, (1) generator, (2) batteries, (3) microfluidic DEP chip.

2.3. *Generator*

The home-made generator was supplied by two 9 V batteries in parallel. Its maximum output was about 2.3 V_{rms}. The following frequencies could be chosen: 100 kHz, 500 kHz, 1 MHz, 5 MHz and 10 MHz.

2.4. *Microparticles*

Polystyrene beads of different diameter (Polyscience Inc., USA) were suspended in Cytocon II buffer (Evotec Technologies GmbH, Germany) which has an electric conductivity of about 300 mS / m.

2.5. *U-937 cells*

U-937 human lymphoma cells (DSMZ GmbH, Braunschweig, Germany) were cultivated in Hepes-buffered RPMI 1640 medium (Biochrom AG, Berlin, Germany) with stabilized L-glutamine, 1% gentamycin, 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. Before the experiments, the cells were transferred by centrifugation washing into Cytocon II buffer (Evotec Technologies GmbH, Germany) at a final density of 1.8×10^6 cells / ml.

2.6. *Blood sample*

75 µl of peripheral blood donated by a healthy donor was mixed with Cytocon II buffer (Evotec Technologies GmbH, Germany) to give a 1 : 100 dilution. This diluted blood was mixed with the suspension of U-937 cells at equal volumes.

2.7. *Preparation of the microsystem*

The chip, the generator and the batteries were fixed to the spinning disk with hot-melt adhesive and tape. Adhesive tape between the disk and the rotor reduced the slip. Wires connected the batteries to the generator and the generator to the chip board. Before each run, the chip was vacuum-dried and pre-rinsed with alcohol to reduce the surface tension. It was then filled with Cytocon II buffer (Evotec Technologies GmbH, Germany) by capillary action. 5 µl of the sample were placed at its inlet on a groove in the board. The channel in- and outlets were sealed with a fast curing silicone rubber (Coltène GmbH & Co. KG, Langenau, Germany). Finally, the voltage was supplied to the appropriate DEP elements by means of the microswitches on the chip board.

3. Results

A first set of experiments served to determine the optimal centrifugation speed. Due to safety considerations, the rotation frequency was limited to a maximum of 30 Hz. For the separation tests described below, rotations of up to 25 Hz proved sufficient. At frequencies of and below 5 Hz, deviations in the synchronization between the rotation and the image acquisition became intolerable.

The first samples tested were suspensions of artificial polystyrene beads. At rotation frequencies between 5 Hz and 15 Hz, particles of 6.5 µm, 9 µm and 15 µm diameter could be successfully manipulated by DEP at all frequencies delivered by the generator. In particular, they were deflected by a cascaded microelectrode arrangement into the side channel of the branched Y-channel (Fig. 3(a), (b)). Additionally, non-standard DEP deflectors were tested (Fig. 4). Bow-shaped microelectrodes effectively aligned the particles on one side of the channel in preparation of a downstream deflection into the side channel (Fig. 4(a)).This also worked with a slant deflector (Fig. 4(b)). As described above, DEP elements usually consist of equally shaped microelectrodes on the top and bottom of the channel. Thus, a vertical electric "wall" extends between them. In the slant deflector, the microelectrodes on both layers are of different size, so that the electric field between them is not vertical but slanted. It deflection efficiency appears slightly reduced near the electrode tips that are furthest apart.

While problems induced by leakage were comparable to the situation in standard pumped LOCs, we encountered unique adverse effects of air bubbles trapped in the fluidic system during filling. In contrast to pumped microfluidic systems, where gas bubbles are often stationary or move in the same direction as the particles, they rise in centrifugation, i. e. move centripetally. Thus, their directionality is opposite to that of the particles and tends to disturb the flow of the latter. Special care has, therefore, to be taken to avoid bubbles in the centrifugation set-up.

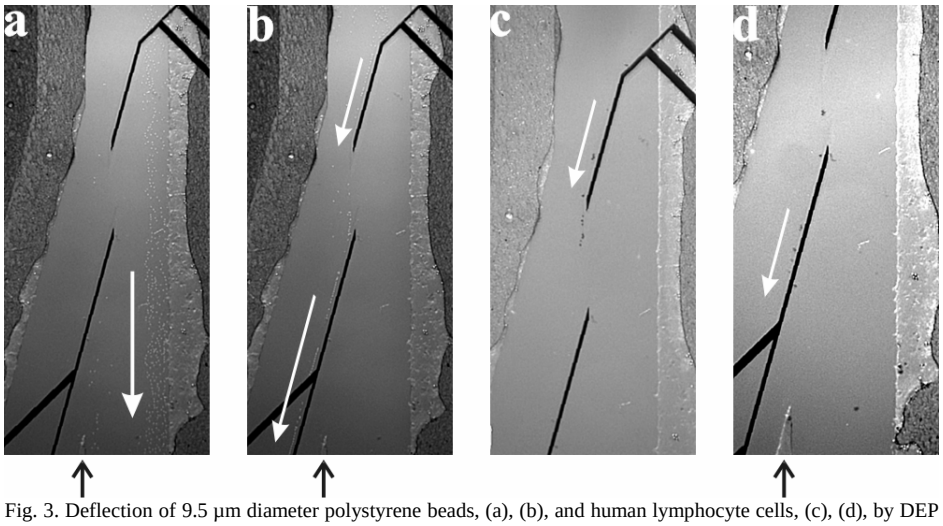

Fig. 3. Deflection of 9.5 µm diameter polystyrene beads, (a), (b), and human lymphocyte cells, (c), (d), by DEP in the centrifugation set-up. The axis of rotation (10 Hz for beads, 19 Hz for cells) is above the images, so that the particles move from top to bottom. Particle trajectories indicated by white arrows. Black arrows below images mark "headlands" of branching. (a) When the microelectrodes are off, polystyrene beads move straight along the channel. (b) Energizing the electrodes results in deflection of beads into side channel (10 MHz, 0.8 V_{rms}). (c) Deflection of cells by the first and (d) second DEP electrode into the side channel (500 kHz, 0.8 V_{rms}).

As particle manipulation was accomplished in the polystyrene beads experiments, we

performed analogous tests on human lymphocyte cells. The dielectric properties of animal cells are similar to those of the surrounding liquid if compared to the case of artificial beads. Therefore, the DEP forces exerted on the cells are weaker because DEP relies on the dielectric contrast between different media **[Pohl, Duschl]**. Nevertheless, the cells were also sorted into either of the two branch channels, depending on the voltage of the deflector electrodes (Fig. 3 (c), (d)). The electric field frequency had to be reduced in order to avoid dielectrophoretic attraction of the cells to the microelectrodes **[Gascoyne, Yang]**. Finally, the U-937 cells were mixed as described with diluted human whole-blood and introduced into the chip (data not shown). At a rotation frequency of 25 Hz, it became possible to separate the added lymphocytes from the erythrocytes on the blood sample by deflecting the former into the side channel while the latter pass the electric field barrier between the DEP microelectrodes unaffected. As DEP forces scale with the particle volume, the larger lymphocytes experience a stronger DEP force than the smaller erythrocytes. In summary, the combined DEP / centrifugation approach was found to be capable of separating cells from a mixed suspension into two different fluidic outlets.

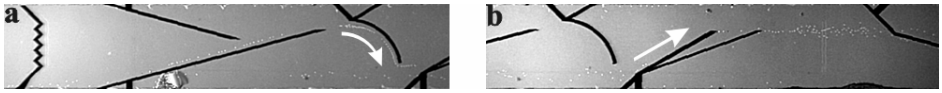

Fig. 4. Additional deflection elements. (a) Bow-shaped electrode. (b) Slant deflector. The particle trajectories are emphasized by the white arrows. The electrode widths are 20 µm. Particle diameter 9 µm. Electric field parameters: 0.8 V_{rms}, 10 MHz.

The lower DEP forces in the cell experiments revealed another centrifugation-specific effect. In routine LOC designs, the heat transferred to the fluid by the electric field between the microelectrodes dissipates by conduction to the channel walls. In extreme cases, it may lead to local convection in proximity to the electrodes **[Gascoyne, Müller]**. In centrifugation, however, the warmer liquid rises, i. e. it moves along the channel towards the axis of rotation. This may interfere with the intended function. Fortunately, various measures can be taken to avoid excessive heating, e. g. a prudential electrode design and a careful choice of voltage and electric medium conductivity.

4. Discussion

In a number of tests with different particles and live cells, this report shows the biological and medical relevance of the novel separation method. We deflected artificial polystyrene beads as well as human lymphocytes into any of two branches of a Y-shaped separation channel. Different DEP elements were tested and found to be fully functional in combination with centrifugation: various deflectors, bow-shaped electrodes and a slant deflector. A future extraction module will enable the recovery of the two cell samples after separation. The described approach joins the stress-free cell handling by DEP with the ease of manipulation by centrifugation.

Moving the cells through the separation channel by centrifugation is well-suited to a sorting process based on DEP because centrifugation can effect the appropriate velocity

range of the particles along the channel long axis. This combination has several advantages over existing techniques. Firstly, the suspension fluid surrounding the cells is itself at rest. By that, the sample consumption is extremely low. A minute droplet placed at the channel inlet suffices for processing. Secondly, the centrifugation supersedes external pumps. Many LOC microchips need a fluidic periphery that is orders of magnitude larger than the chip itself - in size as well as in cost. At the same time, changes in the sample composition, as in electroosmosis, are avoided.

A drawback of the proposed method can be mainly seen in the high demands regarding the imaging system. At the highest rotation frequency, the middle of the separation channel moves at about $7\ \mathrm{m/s}$ or $25\ \mathrm{km/h}$. Thus the need for short exposure times which, in turn, necessitate a strong illumination. This makes fluorescence imaging somewhat more difficult than in resting chips. In contrast, the current limitations regarding the output of the generator can easily be overcome by remote control and triggering.

After equilibration of the centrifugal and friction forces, spherical particles with the density ρ and the radius r move with the velocity v through the channel filled with a fluid of viscosity η where $v = \frac{8 \cdot \pi^2 \cdot f^2 \cdot d \cdot \rho \cdot r^2}{9 \cdot \eta}$. d denotes the distance from the axis of rotation and f the rotation frequency. Note that, in contrast to conventionally pumped particles, their velocity in the system shown here is not constant but linearly depends on d. While the particles move along the separation channel, this distance d continuously increases and so, consequently, also does their velocity v.

The relationship given in the previous paragraph hints at another advantage of the described set-up. DEP is a volume-dependent method and this fact has been exploited for the separation of differently sized particles in various instances [Kentsch, Duschl, Müller]. DEP, however, only deflects the particles perpendicularly to the channel long axis. As the above equation shows, in the combined DEP / centrifugation approach, their movement along this axis also depends on their size, namely on r^2. Therefore, larger cells pass the channel faster. This opens up the possibility of an additional separation step which can never be achieved in conventional systems where all particles are dragged along by the same fluid flow and cannot overtake each other.

In addition, the absence of a fluid flow in the described approach resolves problems associated with the Poiseuille profile that occurs in standard LOC microchannels. In a parabolic profile, particles that sediment to the bottom reach areas of lower velocity and tend to stick to the channel surface. Secondly, large elastic particles are transported to the channel centre by the Fåhræus-Lindqvist effect. Both effects are prevented by centrifugation as a particle's velocity is independent on its position with regard to the channel cross section.

The results presented here indicate two interrelated shortcomings of the current set-up. As soon as the rotation is started, the particles in the sample begin migrating through the channels. However, due to the inertia of the system, the selected rotation speed is only

reached after a few seconds. Thus, a fraction of the particles may escape the optical control and cause a contamination by not reaching the correct branch channel. This problem is related to the fact that the voltage on the DEP elements can only be switched between experimental runs. A remote-controlled voltage supply would of course alleviate this issue as a DEP elements could be integrated which would hold back all particles at the entrance of the separation channel until switched off. This is currently addressed in the further development of this system.

For completeness, it should be noted that due to the physical equivalence of inertia and gravitation, the particles can of course also be moved through the separation channel by sedimentation. Corresponding tests already proved successful but despite the more difficult optical set-up, centrifugation has the strong advantage that the particle velocity can be tuned. The only option to achieve this in sedimentation would be by laboriously adjusting the fluid density.

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