LAB-ON-CHIP-BASED CELL SEPARATION BY COMBINING DIELECTROPHORESIS AND CENTRIFUGATION*

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Cell-based approaches in medicine, biotechnology and in pharmaceutical research offer unique prospects to cope with future challenges in the field of public health. Stem cell research, autologous cell therapies and tissue engineering are only a few possible key applications. Progress in these fields will depend on the successful implementation of versatile and flexible tools for the gentle manipulation and characterization of cells. In recent years, we and others have introduced microfluidic lab-on-chip systems that include dielectrophoretic elements for the contact-less handling and the analysis of cells. Here, we present results that were obtained by combining our lab-on-chip devices with a low-cost centrifugation stage for the efficient and gentle separation of microparticles and live human cells. Our approach is supposed to overcome limitations that arise from the use of bulky and expensive external pumping stages.

Keywords: Cell processing; cell manipulation; microelectrodes; microfluidics; centrifugation; dielectrophoresis.

1. Introduction

The efficient and at the same time gentle separation, washing or sorting of small numbers of valuable biological cells and bioparticles such as viruses are key tasks in modern cellular biotechnology, in biomedical processing and in pharmaceutical research. Present needs concern the analysis of blood samples whereas future demands involve the exploitation of stem cells towards the development of cell therapies and of artificial tissues. For critical applications, standard existing methods such as centrifugation or magnetic bead methods cannot be used in a straightforward way if the number of available cells is tiny or the cells are fragile. In recent years, it has been demonstrated

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that with the combination of dielectrophoresis (DEP) and microfluidics one is able to process small numbers of cells in an efficient and gentle manner. In a lab-on-chip architecture, contact-less cell analysis, cell separation, cell washing and cell fusion has been performed with a variety of different cell types[1-6].

In dielectrophoresis, the polarizability contrast between a cell and its surrounding medium can be exploited to exert forces on it using high frequency electromagnetic fields. If the polarizability of the cell is lower than that of the medium, a translational force acts towards regions of reduced field strength. Radio-frequency electric fields are employed, as they do not interfere with physiological processes in the cell. As the strength of the forces is proportional to the gradient of the field, microelectrodes are required in order to produce sufficiently steep gradients on the scale of the dimensions of the object to be manipulated. Through a skilful arrangement of electrodes in a lab-on-chip system, field distributions can be generated that allow the contact-less trapping or deflection of cells in the laminar flow in a microchannel. Multiple inlets and outlets are used for the production of parallel flows of several different fluids in one channel. Together with deflection elements, such arrangements allow the gentle separation, washing and - as the dielectrophoretic force scales roughly with the volume of the objects - the sorting of cells of different sizes[7,8].

The fluidic transport, in general, relies on external pumping using rather bulky syringe pump-based stages as they produce sufficiently pulse-free flows. The limitations of this approach are obvious: firstly, such pump systems are rather expensive; secondly, the size of the stages prevents the parallel use of several chips as tubing length must be kept as short as possible; and thirdly and most importantly, the pressures that are required to pump medium through microchannels are enormous and rise steeply with reduced channel cross sections. Alternative approaches for pumping that are compatible with the chip architecture are electroosmosis and electrohydrodynamic (EHD) pumping based on traveling electric fields (TW). However, electroosmosis requires high electric field strengths that cause harming of cells mainly through electrochemical effects. EDH pumping is still in its infancy and not sufficiently robust yet to be employed in a working instrument[9].

In this contribution, we present a novel approach that overcomes many limitations due to external pumping. It is based on the combination of low-speed centrifugation and lab-on-chip technology. Centrifugation was previously employed for performing chemical processes that included the effective mixing of different liquids, the chemical reaction of two compounds and finally the analysis of the product[10,11]. The small sample volumes resulted in short processing times.

Here, we demonstrate that by fixing the DEP chip onto a low-cost spinning disk, suspended particles can be transported along well-defined trajectories through a microchannel and separated with DEP electrodes. To evaluate the separation power of these devices, we used polystyrene particles of defined size as well as live human cells.

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 microelectrodes on both slides precisely above each other. Usually, the microelectrodes on both the top and bottom of the channel are identically shaped, so that a vertical electric "wall" extends between them. The only exception is the slant deflector in which the microelectrodes on both layers are of different size. Thus, the electric field between them is not vertical but slanted (cf. Fig. 4b). In summary, 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.
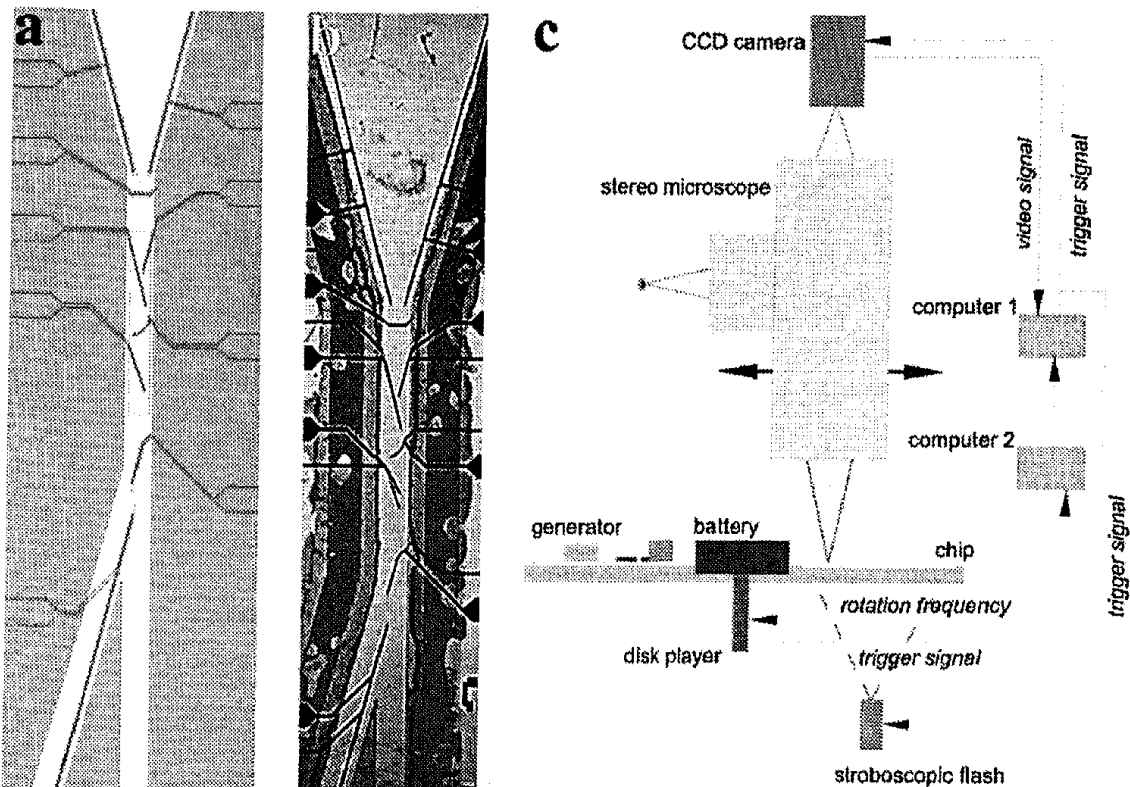

Fig. 1. (a) Design and (b) photograph of the Y-shaped separation channel with one inlet (top) and two outlets (bottom). In this orientation, the axis of rotation is above the images. Before the branching, the channel width is 600 μm. The microelectrodes are processed on both the channel top and bottom 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. 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 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.
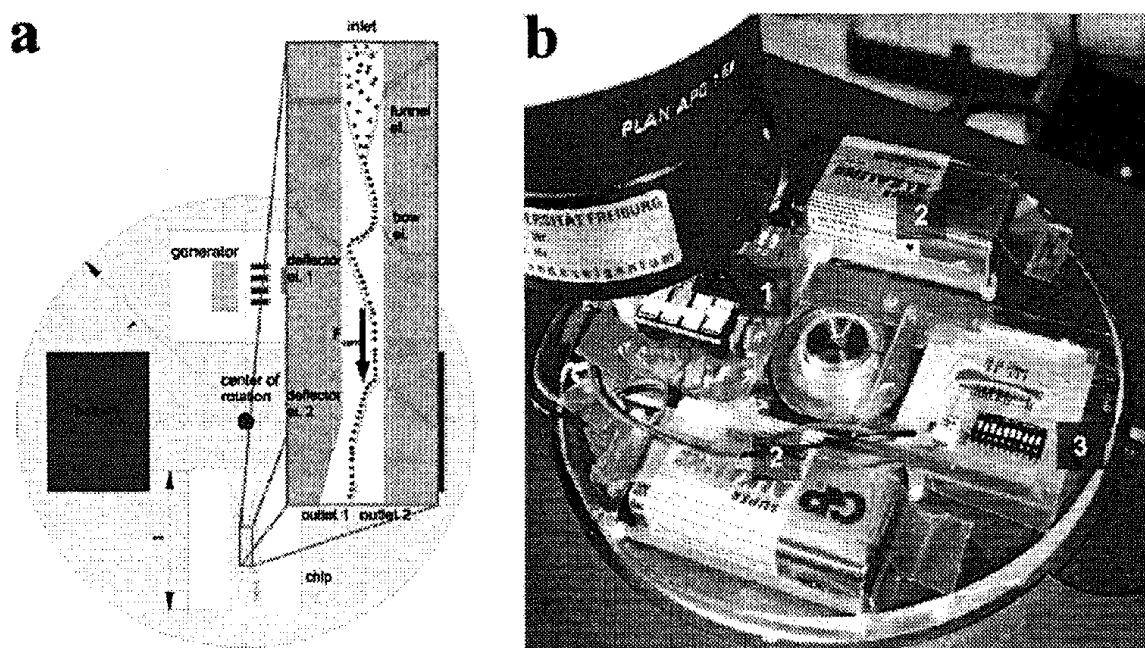

Fig. 2. (a) Scheme of the DEP / centrifugation disk with a magnified view of the microchannel. (b) Photo of the centrifugation disk. All parts are fixed with hot-melt adhesive and tape, (1) generator, (2) batteries, (3) microfluidic DEP chip.

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

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. 3a, 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. 4a).This also worked with a slant deflector (Fig. 4b). Its 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.
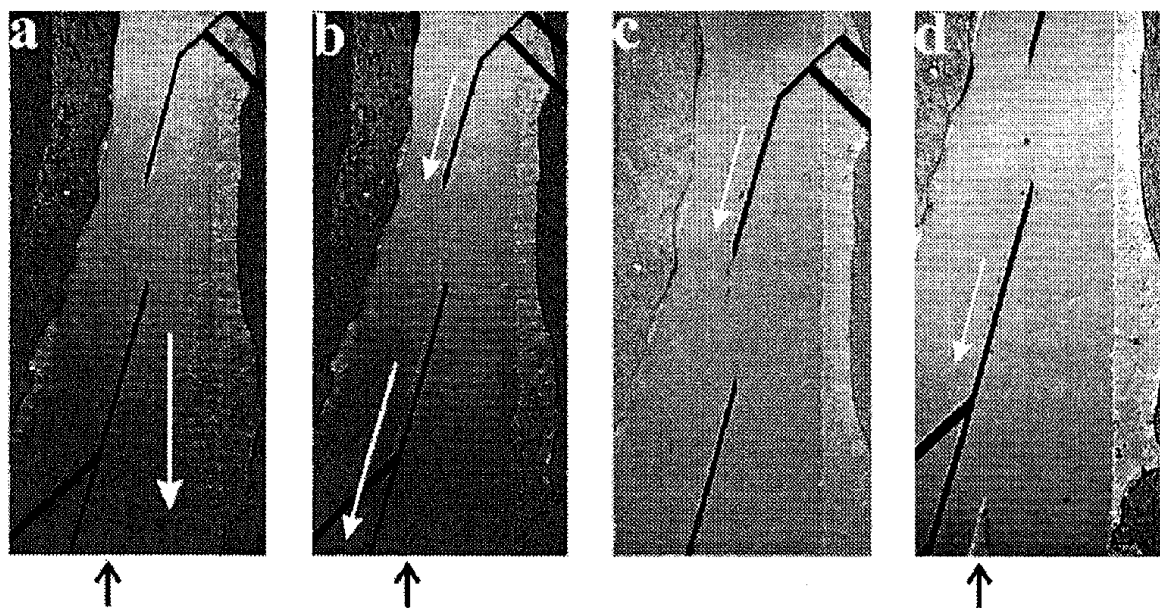

Fig. 3. Deflection of 9.5 μm diameter polystyrene beads, (a), (b), and human lymphocyte cells, (c), (d), by DEP in the centrifugation set-up. The axis of rotation (10 Hz for beads, 19 Hz for cells) is above the images, so that the particles move from top to bottom. Particle trajectories indicated by 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[4, 7]. Nevertheless, the cells were also sorted into either of the two branch channels, depending on the voltage of the deflector electrodes (Fig. 3c, d). The electric field frequency had to be reduced in order to avoid dielectrophoretic attraction of the cells to the microelectrodes[1, 3]. 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[3,5]. 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 m / s or 25 km / h. Thence 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 force F_Z and the friction force F_R, spherical particles of the density ρ move with a constant velocity v through the channel which is filled with a fluid of viscosity η. These forces are given by $F_Z = m \cdot (2 \cdot \pi \cdot f)^2 \cdot d$ and the Stokes drag $F_R = 6 \cdot \pi \cdot \eta \cdot r \cdot v$. Here, r is the particle radius, d denotes the distance from the axis of rotation and f the rotation frequency, while m is the particle mass which can be expressed by $m = \rho \cdot V$, where V is the particle volume: $V = \frac{4}{3} \cdot \pi \cdot r^3$. Setting

$F_R = F_Z$ yields $v = \dfrac{8 \cdot \pi^2 \cdot f^2 \cdot d \cdot \rho \cdot r^2}{9 \cdot \eta}$. 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[4-6]. 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.

Acknowledgements

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