Bioelectrochemistry 73 (2008) 123–128

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Dielectrophoretic sorting on a microfabricated flow cytometer: Label free separation of *Babesia bovis* infected erythrocytes

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ARTICLE INFO

Article history:
Received 31 May 2007
Received in revised form 12 March 2008
Accepted 7 April 2008
Available online 18 April 2008

Keywords:
Dielectrophoresis
Babesia bovis
Infected erythrocytes
Microfabricated flow cytometer
Label-free sorting

ABSTRACT

Dielectrophoresis is a method that has demonstrated great potential in cell discrimination and isolation. In this study, the dielectrophoretic sorting of normal and *Babesia bovis* infected erythrocytes was performed using a microfabricated flow cytometer. Separation was possible through exploitation of the dielectric differences between normal and infected erythrocytes, essentially due to the higher ionic membrane permeability of *B. bovis* infected cells. Sorting experiments were performed inside a microchip made from Pt microelectrodes and SU-8 channels patterned on a glass substrate. Optimum cell separation was achieved at 4 MHz using an in vitro culture of *B. bovis* suspended in 63 mS/m phosphate buffer and applying a sinusoidal voltage of 15 V peak-to-peak. Normal erythrocytes experienced stronger positive dielectrophoresis (pDEP) than *B. bovis* infected cells, moving them closer to the microelectrodes. Under these conditions it was possible to enrich the fraction of infected cells from 7 to 50% without the need of extensive sample preparation or labelling. Throughout the experiments very few microliters of sample were used, suggesting that this system may be considered suitable for integration in a low-cost automated device to be used in the in situ diagnostic of babesiosis.

1. Introduction

Dielectrophoresis (DEP) is a well known method for manipulation of dielectric particles such as DNA, proteins and cells [1].

In the past few years, there has been extensive research in the manipulation and analysis of biological cells at the microscale and a number of microelectromechanical systems (MEMS) using DEP for selective trapping, manipulation or separation of cells was described [1–4]. The goal has been the development of automated single-cell manipulation and analysis systems to be used in research fields such as immunology, developmental biology, tumour biology and parasitology, just to name a few [5].

DEP is defined as the translational motion impaired on uncharged cells or particles, as a result of the polarisation induced by non-uniform electric fields [6]. Using this method, cells can be manipulated to higher and lower electric field regions by means of polarisation forces that induce what is designated as positive and negative dielectrophoresis (pDEP and nDEP), respectively [1].

The dielectrophoretic force on a cell can be calculated using the following formula:

$$\overrightarrow{F_{\mathrm{DEP}}} = 2\pi\varepsilon_m r^3 \mathrm{Re}\left[\overline{K}_{\mathrm{CM}}(\omega)\right] \nabla E^2_{\mathrm{RMS}} \quad (1)$$

where ε_m is the permittivity of the medium, r the radius of the cell, Re $[\overline{K}_{\mathrm{CM}}(\omega)]$ the real part of the Clausius–Mossotti (CM) factor and ∇E_{RMS} the gradient of the root mean square value of the electric field. The CM factor $\overline{K}_{\mathrm{CM}}$, a complex number, can be written as:

$$\overline{K}_{\mathrm{CM}} = \frac{\overline{\varepsilon}_c - \overline{\varepsilon}_m}{\overline{\varepsilon}_c + 2\overline{\varepsilon}_m} \quad (2)$$

where $\overline{\varepsilon}_c$ and $\overline{\varepsilon}_m$ are the complex permittivities of the cell and medium, respectively, and $\overline{\varepsilon}=\varepsilon-(j\sigma/\omega)$ with σ the conductivity, ε the real permittivity, ω the angular frequency of the applied electric field and $j=\sqrt{-1}$.

The value of $Re[\overline{K}_{\mathrm{CM}}(\omega)]$ varies depending on whether the cell is more or less polarisable than the surrounding medium. If $Re[\overline{K}_{\mathrm{CM}}(\omega)]$ is positive, cells are attracted to regions of higher field strength (pDEP) whereas the opposite induces the repulsion of cells from these regions (nDEP) (Fig. 1).

As a result, the dielectrophoretic force is dependent on the dielectric properties and size of the cell, frequency of the applied electric field and conductivity and permittivity of the medium where the cells are suspended. A careful selection of these parameters can induce cell movement in specific directions according to their dielectrical properties, thus enabling cell manipulation or separation. In this study dielectrophoresis was applied in the separation of *B. bovis* infected erythrocytes. *B. bovis* is an intraerythrocytic tick-borne protozoan parasite that infects bovine erythrocytes and is responsible for large

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doi:10.1016/j.bioelechem.2008.04.018

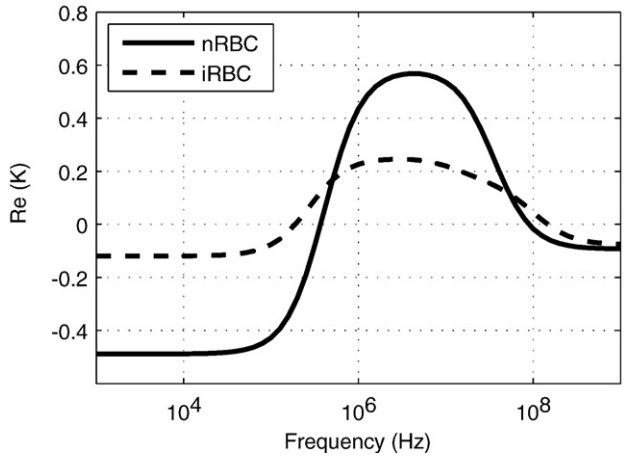

Fig. 1. Plot of the real part of the Clausius–Mossotti factor for normal and *Plasmodium falciparum* infected red blood cells (nRBCs and iRBC, respectively). Based on data published by others [5]. Differences in the two curves allow for separation of the two populations. *B. bovis* probably induces similar changes in cellular dielectric response, although the magnitude could be less since the changes in the erythrocyte aren't as severe as in Malaria, where the host cell experiences a significant change in the cytoskeleton (haemoglobin being converted to hemozoin).

economic losses in cattle industry. This parasite poses particular problems in tropical and subtropical regions worldwide and the major symptom observed in the host animals is anaemia, owing to extensive haemolysis induced by liberation of the parasites from the host cells [7].

Purification of *B. bovis* infected erythrocytes is fundamental for the biochemical and physiological study of the parasite. However, the methodologies currently described and used to discriminate and separate *Babesia* spp. infected erythrocytes have the drawback of requiring time-consuming sample preparation [8,9] and expensive reagents or equipment [10]. Additionally, in contrast to other well-known cell sorting methods, such as fluorescence or magnetic activated cell sorting (which can both be incorporated in MEMS) [11,12] DEP does not require any cell modification by staining with dyes or antibodies. The technique is advantageous because it is non-invasive and allows the discrimination and sorting of *B. bovis* infected erythrocytes from normal cells only by taking advantage of the cellular modifications induced in the membrane of parasitic cells. It is known that, after invasion and while *B. bovis* merozoites develop inside the erythrocyte, the plasma membrane of the infected host cell is structurally [13] and antigenically [14–16] altered. These changes produce alterations in the electrical properties of the infected cells, a fact that is supported by the increase in membrane permeability of *Babesia* spp. infected erythrocytes [9,17]. Here, the use of these physiological alterations in the discrimination and separation of normal from *B. bovis* infected erythrocytes is reported, using DEP forces produced by application of alternating (AC) fields to electrode arrays integrated in a microfabricated flow cytometer.

2. Material and methods

2.1. Cell suspension preparation

The Mo7 strain of *B. bovis* (kindly provided by Dr. Erik de Vries) was grown *in vitro* according to the Levy and Ristic method [18]. Briefly, *B. bovis* infected erythrocytes were cultivated in a microaerophilus phase (MASP) in 24 well suspension plates, at 10% (v/v) packed cell volume (PCV), and incubated at 37 °C in a 5% CO_2 in air humidified atmosphere. Cultures were maintained in M-199 culture medium (Gibco, 22340-020) supplemented with 50 μg/ml gentamycin (Gibco, 15710-049), 1% (v/v) fungizone (Gibco, 15290-026), 20 mM TES (Sigma-Aldrich, T5691) and 40% (v/v) bovine serum (kindly supplied by Dr. Neto from Estação Zootécnica de Santarém, EZN). Subcultivation was performed by splitting/dilution with fresh normal bovine erythrocytes (collected from healthy donors, with normal MHC and MCV values, into sterile collection tubes containing citrate) and medium, when parasitaemia levels achieved 2 or 3%. Parasitaemia was monitored by microscopic examination of Giemsa stained thin smears under a 100× microscope oil objective. *B. bovis* cultures are maintained in non synchronized state, as so far no synchronized cultures are available when cultivating this parasite species, contrarily to the *in vitro* cultivation of Plasmodium spp. *in vitro*.

For the cell sorting experiments, bovine erythrocytes were isolated from bovine whole blood by centrifugation at 2100 rpm and washed with PBS (Sigma-Aldrich, P4417) at 1.4 S/m. During this centrifugation step, bovine erythrocytes were separated from the other components of bovine blood, such plasma, white blood cells and platelets. Erythrocytes were transferred to 0.22 μm filtered low conductivity PBS (pH 7.4 and conductivity range of 10 to 86.7 mS/m); a step which is necessary in order to assure that, when applying electric fields inside the microchip, the electric current remains low and the temperature does not rise to a great extent, eventually causing damage to the cells and electrodes.

Low conductivity PBS was prepared by lowering normal PBS conductivity by dilution in distilled water (conductivity ~5.5 μS/m). Additionally, in order to maintain normal osmolarity values for the cells, a sucrose solution of 9.53 g/100 ml was prepared and PBS was added to the PBS solutions until the desired conductivity level was achieved. Conductivity levels were monitored using a conductivimeter (Radiometer CDM210).

In order to minimize cell adhesion to the microchannel inner surface, 0.1% (v/v) of bovine serum albumin (BSA) was added to these solutions. Cell suspensions from the *in vitro* cultures were prepared by centrifugation at 2100 rpm, using 5 to 10% parasitaemia levels and diluted in the previous solutions at a final density of 10^5 cells/ml.

To quantitatively detect the degree of separation, a fluorescent dye was used in the experiments to facilitate tracking of the infected erythrocytes inside the microfabricated sorting device. Cells were labelled with 25 μg/ml of ethidium bromide (Qbiogene, ETBC1001), a nucleic acid intercalator. Label-free sorting experiments were also performed where the fraction of sorted cells was stained afterwards. Viability tests with 6-carboxyfluorescein diacetate (CFDA; Sigma-Aldrich, cat. no.C5041) were carried out before and after the dielectrophoretic sorting, in order to evaluate cells sensitivity to the electric field, as previously described [19].

2.2. Device fabrication

As schematically shown in Fig. 2 (top left and right), the microfabricated chip used contains an array of 15 pairs of metal electrodes designed along the sides of a microchannel. These electrodes (20 nm Ti for adhesion, 200 nm Pt) were patterned by a standard lift-off process on a 550 μm float glass wafer. In order to form the lateral and middle channels on top of the electrodes, a 20 μm layer of SU-8 was patterned by photolithography. The central channel obtained (where separation is performed) is 40 μm wide and 1200 μm long. The finished wafer was diced and each individual 15 mm×20 mm chip was reversibly sealed by means of a flat PDMS (a silicone elastomer, poly (dimethylsiloxane)) cover. In previous work, microchip configurations identical to the one described above, containing only 2 pairs of electrodes have been presented [19,20].

2.3. Experimental setup

Prior to chip/PDMS assembly, the PDMS cover was placed in vacuum for 30 min. This treatment was necessary to avoid the trapping of air bubbles inside the microchannels, improving gas evacuation by diffusion into the PDMS. Each microchip was then mounted on a poly(methylmethacrylate) (PMMA) custom-made fluidic

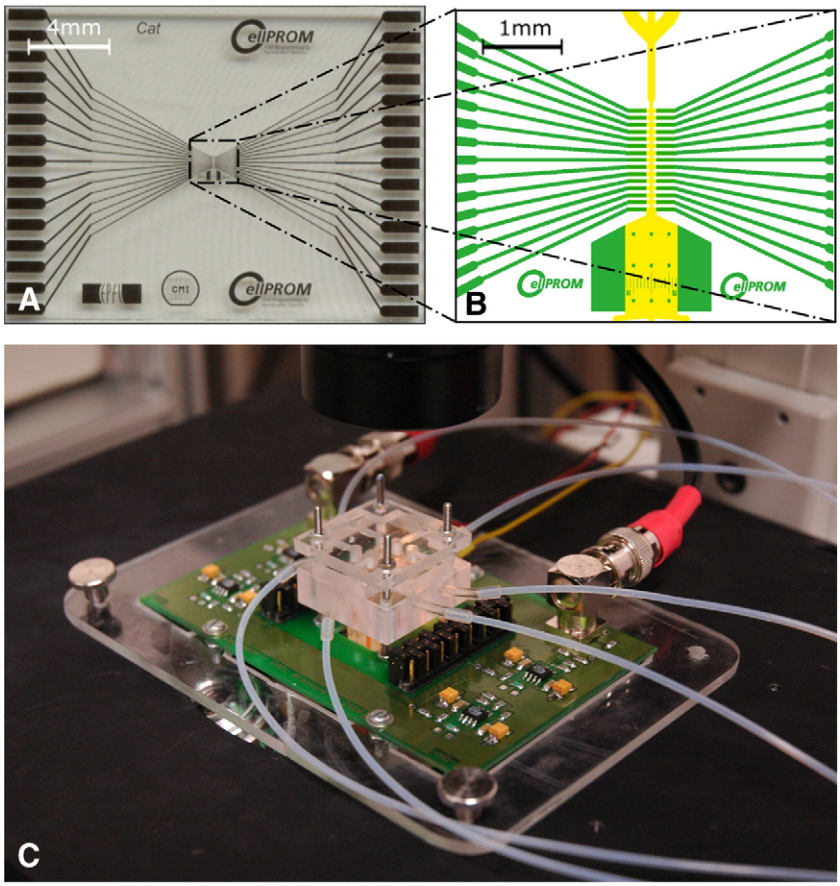

Fig. 2. Microfabricated flow cytometer setup. (A) Chip photograph. (B) Illustration of the electrode pairs along the microchannel. (C) Complete setup, including electrical and pneumatic connections.

interface that included: i) inlet and/or outlet holes for fluid injection and ii) connections to a pneumatic system which has already been presented [21]. The pneumatic system enabled fine control of the cell suspension inside the microfluidic device through pressure driven flow. Fig. 2 (bottom) illustrates the whole structure connected to a printed circuit board (PCB) where the electric interface was made with spring contacts. The PCB board provided amplification and conditioning of the voltage signals applied at the metal electrodes. The whole assembly was mounted on top of an inverted epi-fluorescence microscope (Nikon Eclipse TE-2000-S) for cell visualization inside the device. Before each sorting experiment, the inlets and outlets of the lateral channels were filled with 15 μl of the low conductivity PBS buffer whereas the inlet of the middle channel was loaded with 15 μl of the cell suspension. The top of the wells was sealed with scotch, forming an airtight connection of the wells to the pressure inputs.

In this microfabricated flow cytometer, the cell stream was focused by sheath flow, priming PBS on the lateral channels, and also by electrical focusing. A cell flow of 0.1 μl/min was used and sinusoidal AC electric potentials were applied at each set of electrodes (left and right electrode arrays), in order to perform the electrical cell focusing by nDEP (Fig. 3). In order to avoid the interference between the two electric fields and a resulting beating effect, two different frequencies were used. These sinusoidal focusing signals were designated V_{ref1} and V_{ref2} and were applied at frequencies of 56.8 kHz and 24 kHz and amplitudes of 8.5 and 10 V peak-to-peak (Vpp), respectively.

In order to separate the normal from the *B. bovis* infected erythrocytes, an extra signal of 4–15 Vpp (V_{scan}) was added to V_{ref2}. The amplitude of this signal was adjusted to the highest value that wouldn't enable cell attraction to the electrodes. The frequency was swept from 100 kHz to 20 MHz with the purpose of identifying the optimal frequency for separation of both cell types.

V_{ref2} and V_{scan} signals were generated using two 20 MHz DDS function generators (TTi TG2000), whereas the V_{ref1} signal was generated by a 2 MHz sweep function generator (Escort EGC-3230). Videos of the

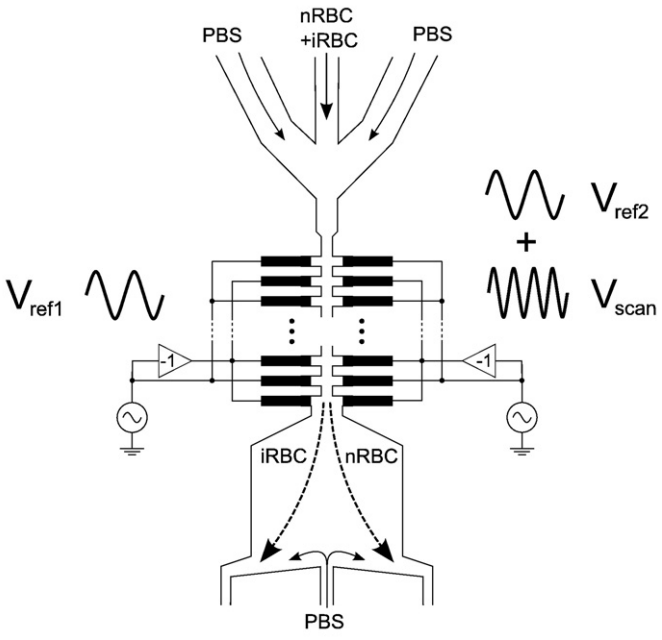

Fig. 3. Schematic diagram showing the applied voltages and cell flows inside the microchip. The PBS lateral inputs on the top of the figure are used for hydrodynamic focusing of the cell suspension, while the PBS input in the bottom is used to aid the separation of both cell types.

separation were acquired using a CCD digital camera (Evolution MP Version 5.0) and image analysis software (Image Pro Plus version 5.1 from Media Cybernetics). Quantification of the separation extent was performed by analysis of the captured video sequences obtained at each sorting experiment.

2.4. Image analysis

A routine for post-processing the captured video sequences was written in MATLAB (The Mathworks, Inc.). The program allowed an estimation of the distribution of normal and infected cells along the channel after sorting, in order to quantify the extent of DEP separation at each frequency.

A thin section of the image sequence, corresponding to the region of the channel after the sorting, was chosen, where both normal and infected red blood cells were counted. This section was always located in the middle channel, after the last pair of electrodes. The background is then estimated and removed from each frame, resulting in a sequence of images that only contained cells crossing the selected section in a specific time frame. For each frame, an image matrix was obtained containing the colour intensity values for each pixel. By adding the intensity values along the direction of the channel, a vector was obtained. By choosing the section thin enough, one can make sure that no cells are superimposed when adding. Assuming that the normal erythrocytes are darker and that the infected (stained with a fluorescent marker) are brighter than the background, it is possible to identify each cell in the obtained vector. By comparison of the brightness values with threshold values previously chosen, the program calculates the position of the cells in the channel and stores them. The cell positions obtained for each frame were then integrated in time, resulting in a distribution of the cells in the microchannel for a given sequence of frames. Assuming a normal distribution of the cells along the channel, Gaussian curves were fitted to the obtained data and normalized, resulting in a probability density function for each cell type. This allowed the comparison of the cell separation using different separation parameters (V_{scan} frequency and medium conductivity).

3. Results and discussion

Post-processing of the video sequences captured for each sorting experiment allowed an evaluation of the separation extent between normal and *B. bovis* infected erythrocyte populations. As the DEP force depends on the CM factor and this on the conductivity and permittivity of the cell and medium, the sorting experiments were performed using different medium/buffer conductivities (~10 to 86 mS/m range). The extent of separation was evaluated by means of the probability of each cell type to cross a specific region of the chip microchannel (region after the electrodes, where the cells are directed to the respective outlets).The results were displayed in Gaussian curves *versus* the distance cells occupied in the microchannel after sorting. Therefore, a high degree of separation meant that a high proportion of normal erythrocytes would be seen in a specific region of the channel, whereas the majority of *B. bovis* infected erythrocytes would occupy a different region in the same channel.

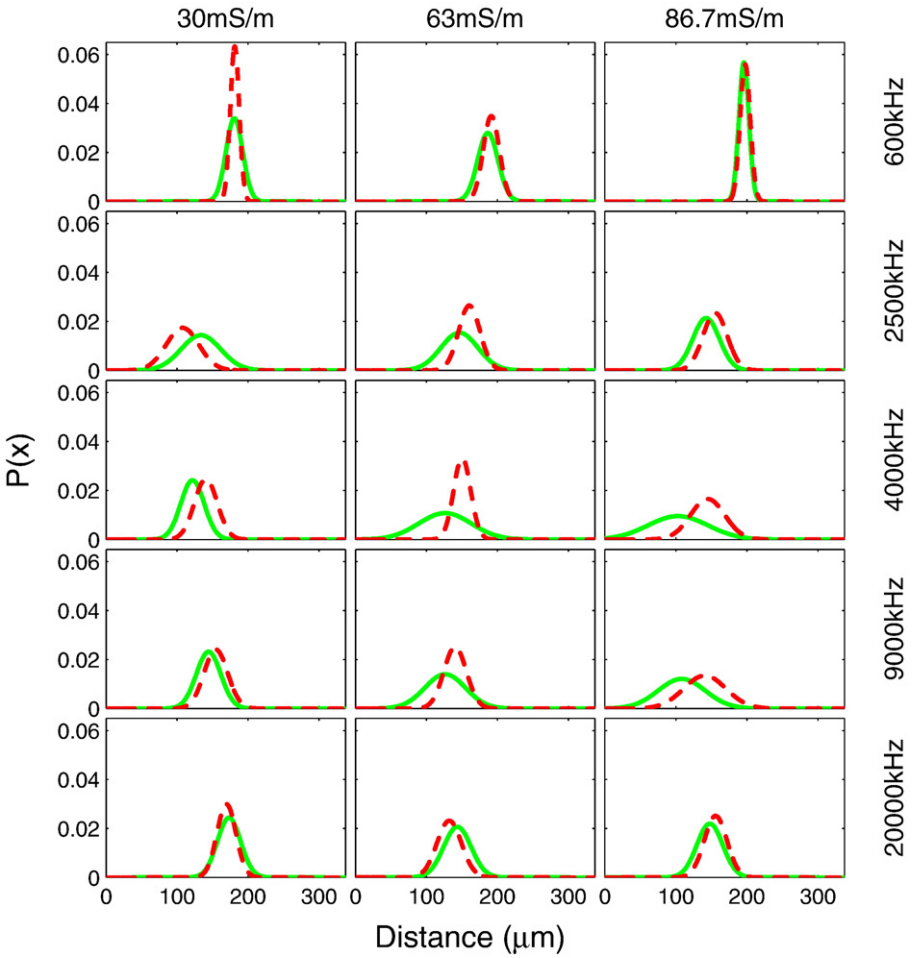

Fig. 4. Probability density functions obtained for the distribution of normal (solid line) and *B. bovis* infected red blood cells (dashed line) along the channel width after DEP separation. Results show sorting performed at 30, 63 and 86.7 mS/m medium conductivities and frequencies ranging from 600 kHz to 20 MHz.

To maximize the separation extent, in addition to changing the electric properties of the medium where cells were suspended, the frequency of the electric field applied was swept from 100 kHz to 20 MHz. Fig. 4 shows the probability distributions for each cell type at different frequencies and medium conductivities.

Note that these plots represent the probability density function for each cell type, and not the relationship between both cell distributions (in the later case it is necessary to take into account the parasitaemia levels). The depicted results illustrate a high degree of separation at 63 mS/m, in comparison to the results obtained at 30 mS/m and 86.7 mS/m, where small separation peaks between normal and *B. bovis* infected erythrocytes were obtained. At the low frequencies of 100–300 kHz (data not shown) and 600 kHz, no separation is observed, meaning that these low frequency electric fields are not capable of discriminating between infected and normal cells.

In order to prevent the adhesion of normal red blood cells to the electrodes, voltage levels were adjusted from 4 to 15 Vpp and separation of each cell type to the respective outlet was possible (Fig. 5A).

Starting from 2500 kHz, a slight deviation between both cell types is observed due to the strong pDEP phenomenon in normal red blood cells. This effect is more prevalent at 63 mS/m. Increasing the frequency to 4 MHz enables higher peak separation. Nevertheless, a further increase in frequency does not produce better separation results. Instead, *B. bovis* infected cells start experiencing pDEP, decreasing the separation efficiency. This effect was observed at frequencies above 9 MHz at all the medium conductivities used.

The highest degree of separation was observed at 4 MHz and 63 mS/m. Using these parameters, and starting with an initial parasitaemia of 7%, it was possible to achieve a final parasitaemia of 50% (Fig. 5B).

A control experiment was performed using the same separation procedure without ethidium bromide labelling. Cells were collected from the outlet, ethidium bromide stained and counted under the epifluorescent microscope. Approximately the same enrichment level was achieved (see Table 1), meaning that the deviation of cells was due to the dissimilar electrical 'phenotype' of normal and *B. bovis* infected cells and not due to labelling.

The use of this microfabricated flow cytometer allowed a ~7 fold enrichment in *B. bovis* infected cells, using just a few microliters of cell suspension and without affecting cell viability (data not shown). As previously mentioned, the cell separation depends on the DEP force exerted, and this is a function of the membrane capacitance, membrane conductivity, and internal conductivity of the cells. The normal and *Babesia* spp. infected cells' membrane conductivities have been measured and are different [9]. This study took advantage of these features in order to perform the label-free separation of both cell types. An increase in separation efficiency might be possible through redesign of the microchip's configuration. For example, immediately after a first sorting step, cell recirculation could be promoted from the outlet to the inlet or subsequent sorting regions could be added to consecutively enrich each cell type fraction.

Table 1
Cell counting of normal (nRBC) and *B. bovis* infected red blood cells (iRBCs) containing samples from both left and right outlet channels and from middle inlet channel for sample priming

Parameter	Middle inlet channel (sample priming)	Right outlet channel	Left outlet channel
# iRBCs	25	74	20
# nRBCs	536	87	257
PPE (%)	7	46	7

Cells were stained after collection from the microchip and counted under an epifluorescence microscope.
The parasitaemia percentage (PPE) is calculated by counting the number of parasitized red blood cells in 1000 cells suspension.

4. Conclusions

Previous studies have shown that it is possible to discriminate between normal and *B. bovis* infected erythrocytes only by their electrical 'phenotype' [19]. However, if in one case these dissimilarities are used for label-free characterization and cell type discrimination, here the different dielectric characteristics were used for cell separation. The differences observed between normal and *B. bovis* infected erythrocytes arise from the changes in membrane and cytosol morphology as a consequence of the high metabolic activity of *B. bovis* infected erythrocytes in comparison to the simpler metabolism and biochemistry of the normal erythrocyte [9].

The miniaturized flow cytometer described proved to be useful in the enrichment of *Babesia* spp. infected cells using very small amounts of sample. Additionally, no label or sample preparation was necessary, proving to be advantageous when compared to the existing methods, where high amounts of sample are needed and several centrifugation steps are essential [8,9]. A parallelism can be made to a hypothetical system where human erythrocytes infected, for example, with *Plasmodium* spp., can be separated from healthy cells, but first isolating the fraction of erythrocytes by centrifugation, and then applying DEP for separation of normal and infected cells. This can be made, because the same antigenic and cytoskeleton changes occur in both cases, but more dramatically in Plasmodium spp., where the production of the pigment hemozoin induces greater host cell modification.

This device has the potential of being incorporated into an integrated lab on a chip (LOC) device that performs cell sorting, cell cultivation and *in vitro* single-cell analysis. Such a LOC device might be used in the diagnostic of babesiosis in a near future. Incorporation of a lysis region after the sorting module used, would allow DNA removal

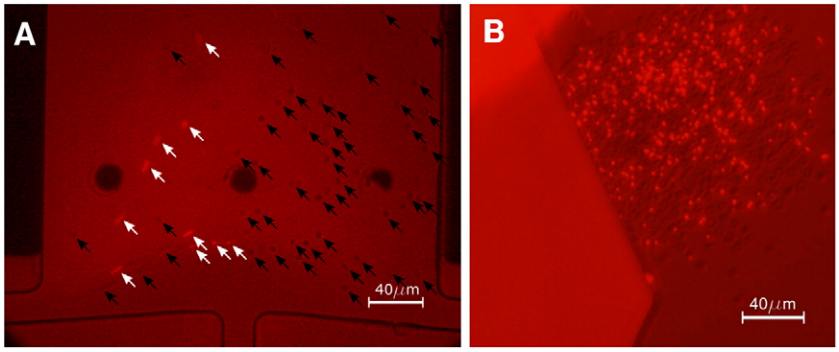

Fig. 5. Dielectrophoretic sorting of normal and *B. bovis* infected erythrocytes at 4 MHz and 63 mS/m phosphate buffer. Red spots are *B. bovis* infected erythrocytes stained with ethidium bromide (25 μg/ml). (A) Cell separation after DEP sorting (region after the electrodes). Arrows were added for clarity, black for normal RBCs and white for infected RBCs. (B) Microfluidic outlet after separation. Parasitaemia percentage was calculated by image analysis and estimated to be 50% at the end of the sorting procedure.

to be used in a hybridization step similar to the reverse line blot technique used for the diagnostic of babesiosis [22]. Such portable device would be suitable for the *in situ* diagnostic of *Babesia* spp. and similar protozoan parasites. Most countries affected by the disease lack the equipment and infrastructures that would allow a rapid and efficient diagnostic and a LOC device would reduce the diagnostics time response, preventing spread of the disease.

Acknowledgements

B. bovis clonal line Mo7 was kindly provided by Dr. Erik de Vries, from the Department of Infectious Diseases and Immunology, Utrecht University, in the Netherlands.

Bovine blood from healthy donors was kindly provided by Dr. Maria Irene Vasques and Dr. João Maria Neto from Estação Zootécnica Nacional (EZN), in Portugal.

This work was supported by the European Community-funded CellPROM project under the 6th Framework Programme, contract No. NMP4-CT-2004-50039.

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