Sensors and Actuators B xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Cell viability assessment by flow cytometry using yeast as cell model

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Cell sorting
Cell counting
Dielectrophoresis
Impedance measurements
Label-free
Viability study

ABSTRACT

This paper reports the new combination of cell sorting and counting capabilities on a single device. Most state-of-the-art devices combining these technologies use optical techniques requiring complicated experimental setups and labeled samples. The use of a label-free, electrical device significantly decreases the system complexity and makes it more appropriate for use in point-of-care diagnostics.

Living and dead yeast cells are separated by dielectrophoretic forces and counted using coulter counters. The combination of these two methods allows the determination of the percentage of living and dead cells for viability studies of cell samples. The device could further be used for sorting and counting of blood cells in applications such as diagnosis of insufficient cell concentrations, identification of cell deficiencies or bacterial contamination. The use of dielectrophoresis (DEP) as sorting principle allows to separate cells based on their dielectric properties in the place of size-based separation, enabling sorting of large panels of cells and separation of infected and non-infected cells of the same type.

1. Introduction

Recent advances in microtechnology have enabled the fabrication of devices integrating fluidic, electrical and optical components for biological applications. These lab-on-chips make use of different techniques enabling sample preparation and analysis. In sample preparation, one important technique is the sorting of cells, which can be performed optically or electrically, using among others dielectrophoresis [1,2], magnetophoresis [3,4], fluorescence-activated cell sorting [5] or optical tweezers [6]. On the other hand, sample analysis can be also integrated on chip, the most common example of which being cell counting. This counting can rely either on optical monitoring of a specific position on the chip [5,7], or on electrical impedance measurements influenced by the cell passage [8–11]. The latter technique is widely known in its coulter counter configuration, first developed by Coulter [12] and later miniaturised by Larsen et al. [13].

This paper presents a lab-on-chip integrating cell sorting and counting abilities, based on dielectrophoresis and coulter counter, respectively. To the best of our knowledge, this is the first example of a fully electric system, which has the advantages of a label-free protocol and a relatively simple experimental setup. This device allows determining the concentration of cell subpopulations in a sample, with applications such as viability studies and differential cell counting for point-of-care diagnosis.

2. Materials and methods

2.1. Chip design, fabrication and packaging

In order to integrate cell sorting and counting, a device is developed using the design shown in Fig. 1, featuring microfluidic channels and electrodes for dielectrophoresis and impedance measurements. The fabrication of the device is described elsewhere [14] and briefly explained here. Platinum electrodes are deposited on a titanium adhesion layer by evaporation and patterned by lift-off. Microfluidic channels are then defined using SU8 photolithography. A silicone elastomer block placed on top seals the fluidic network and allows access to the inlets and outlets via a plastic holder. Pressure-driven flow is regulated by a pressure box, as described by Braschler et al. [15].

2.2. Protocols

Yeast cells (Baker's Yeast, *Saccharomyces cerevisiae*) are obtained from a local grocery store. A mixed population of living and dead cells is prepared at a cell concentration of 1% (w/v). The working solution is a Phosphate Buffer Saline (PBS) solution diluted 30 times to reach a conductivity of 55 mS/m, which is suitable for the cell sorting. Dead yeast cells are obtained by heating a living sample for 30 min at 90 °C, as performed in [2], so that the cell membrane and cell wall become leaky. Other treatments could also be used to kill

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doi:10.1016/j.snb.2009.11.066

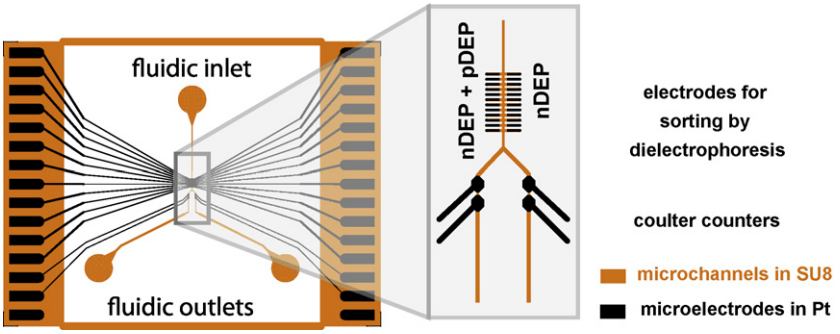

Fig. 1. Schematic of the chip design, including the microfluidic channels in SU8 and the platinum electrodes.

the yeast cells, such as chemical lysis or UV radiation. A standard protocol for exclusion assay is used to stain dead cells with trypan blue for visual identification.

3. Results and discussion

3.1. Cell sorting

After being injected in the inlet, cells flow through two parallel arrays of "liquid electrodes" where voltages are applied as summarized in Table 1. Liquid electrodes are large metal electrodes patterned at the bottom of dead-end chambers positioned perpendicularly to the main channel as defined by Demierre et al. in [16]. Resulting electric fields polarise cells according to their permittivity and conductivity. In an inhomogeneous and time-varying electrical field E, net forces are applied to cells with a time average given by the following equation [17]:

$$\langle F_{DEP}(t)\rangle = \pi\varepsilon_m r^3 \, Re(K_{CM})\nabla|E|^2$$

where ε_m is the permittivity of the medium, r the radius of the particle, and $Re(K_{CM})$ is the real part of the Clausius–Mossotti factor defined as

$$K_{CM} = \frac{\underline{\varepsilon}_p - \underline{\varepsilon}_m}{\underline{\varepsilon}_p + 2\underline{\varepsilon}_m}, \underline{\varepsilon} = \varepsilon - j\frac{\sigma}{\omega}$$

ε being the permittivity and σ the conductivity of the particle and the medium; ω is the angular frequency of the electric field, j the imaginary unit. K_{CM} is bounded between −0.5 and 1, and can give rise to DEP forces in two opposite directions. Namely one refers to positive dielectrophoresis (pDEP) when cells are attracted in regions where the gradient of electric field is large. Conversely, cells are repulsed from those regions in the case of negative dielectrophoresis (nDEP), when cells are less polarisable than the surrounding medium.

The presented chip achieves living and dead yeast cell separation by means of dielectrophoretic forces. Fig. 2 shows the real part of K_{CM} for living and dead yeast cells as a function of the frequency, according to the multi-shell model described in [18]. Negative dielectrophoresis alone is often used for cell manipulation/trapping [19,20], whereas in this work multiple-frequency dielectrophoresis allows separation of different cell types depending on their dielectric properties. In the 200 kHz frequency range, the cell membrane and cell wall are opaque to the electrical field and both living and dead yeast cells experience the same nDEP force, allowing electrical deflection of the cell stream in an equilibrium position to the right. In the 5 MHz frequency range, the cell membrane and cell wall are transparent to the electrical field and the cytoplasm influences the dielectrophoretic force. Due to their resistive cytoplasm, dead yeast cells again undergo nDEP (being thus further pushed to the right), whereas living cells, which have a more conductive cytoplasm, are subjected to pDEP forces attracting them to the left. Further details can be found in [2].

The cell populations focused at different positions are separated in two channels, as shown in Fig. 3. Thanks to the staining of the dead yeast cells with trypan blue, it is possible to visually evaluate the sorting efficiency. Out of 582 cells sorted on the left, only 0.26% were dead cells, whereas 3.69% of the 177 cells deviated to the right were living cells. The majority of the wrong sorting events is due to cell clusters containing both living and dead cells.

Table 1
Amplitude and frequency of the voltages used for cell sorting and counting.

Signals	Amplitude	Frequency
nDEP (left)	3.5 V_{pp}	200 kHz
nDEP (right)	1.2 V_{pp}	200 kHz
pDEP (right)	2 V_{pp}	5.003 MHz
Counting signal	2 V_{pp}	101 kHz

3.2. Cell counting

After sorting, cells are counted by means of a coulter counter by monitoring the current variations occurring at each cell passage through an aperture, across which a constant voltage is maintained. The channel impedance is measured around 100 kHz, where the cell membrane is opaque so that the current can only pass

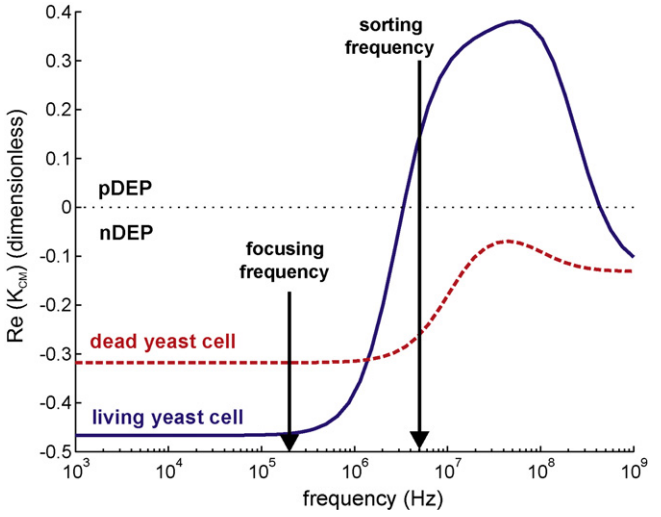

Fig. 2. Variation of the real part of the Clausius–Mossotti factor as a function of the frequency for viable and non-viable yeast cells in low conductivity buffer (55 mS/m). Both cell types experience nDEP at the focusing frequency whereas living cells experience pDEP at the sorting frequency. In a buffer featuring a too high conductivity, living cells would also experience nDEP at high frequencies and such separation could not be performed anymore.

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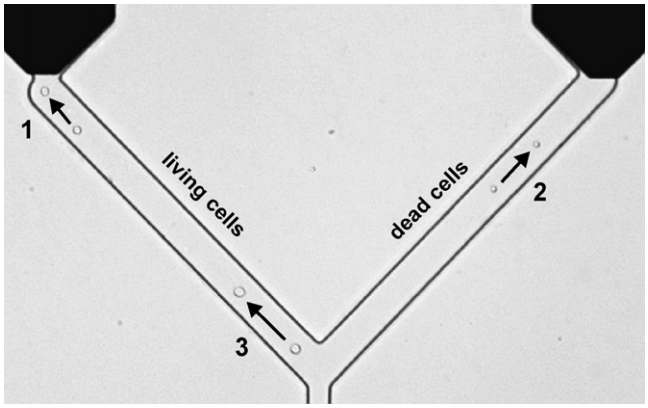

Fig. 3. Superposition of 2 video frames, showing the trajectory of 2 non-colored living cells (left) and a colored dead cell (right) sorted by dielectrophoresis.

around the cell, thereby leading to a more resistive channel. Precise calculations of the impedance variation can be found in [21]; a rough estimation based on the cell size (sphere with 4 μm radius), solution conductance (55 mS/m) and channel geometry suggests impedance variations of about 1% over a 1 MΩ channel resistance, which can be detected with an appropriate device.

Counting is performed using a commercial impedance spectroscope (HF2-IS, Zurich Instruments AG, Switzerland). Voltage signals are applied to coulter channels, and resulting currents are converted into voltages by a differential current probe (HF2 Current Amplifier, Zurich Instruments AG). This current probe features an adjustable input impedance chosen at 1 MΩ to match the Coulter electrodes' impedance which, combined with a 10 times amplification, gives an equivalent transimpedance gain of 10 MV/A. The signals are then demodulated with a lock-in amplifier integrated in the HF2IS. The absolute impedance is recorded for both channels simultaneously, the passage of cells in a coulter counter causing peaks in the corresponding signal, as shown in Fig. 4.

The excellent signal-to-noise ratio obtained allows automated counting with very simple signal processing. As cell sizing is not aimed here, no calibration of the individual channel sensitivity has been made.

3.3. Application of the device for viability measurement

The developed device aims at determining the viability of a cell sample. Fig. 5 shows different measurements of the viability of the same sample, either made by visual observation of the cells or by analysis of the counting signal. The first column shows the viability

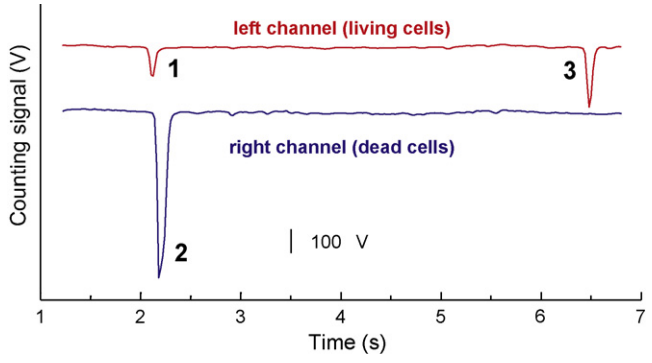

Fig. 4. Signal recorded from the cell counting electrodes, featuring peaks corresponding to cells passing in the channel. As it comes from the measured current, the signal is inversely proportional to the channel impedance. The data shown here corresponds to the cells present in the sorting image stack shown before.

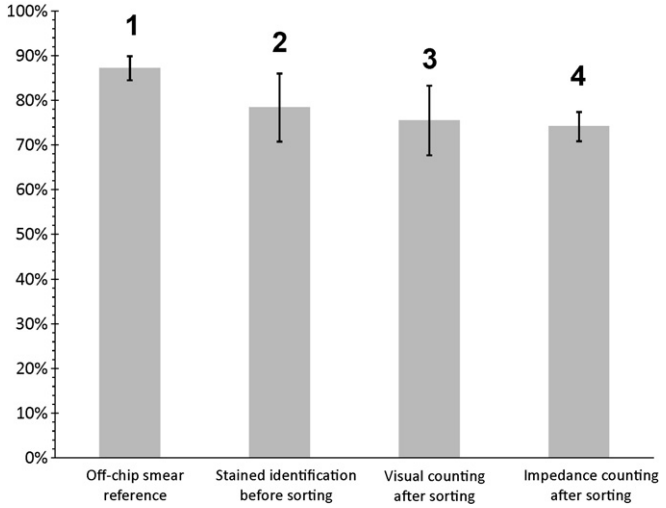

Fig. 5. Comparison of different methods for viability measurements, from traditional counting off-chip using smears to final counting on chip. Intermediate columns are the viability of the sample arriving in the sorting channel, based on their color, and visual counting of the cells sorted in the different channels.

determined off-chip by counting living and dead cells on traditional smears, giving a reference for the real sample viability. The second column comes from the visual identification of living and dead cells arriving in the sorting channel using the trypan staining. The sample is then sorted in the two channels, where the cells can be counted visually under a microscope (third column) or electrically using the coulter counters (fourth column).

This graph shows the three sources of errors in the viability measurement: the sample arriving in the sorting channel not representative of the full sample (difference 1 – 2), the cells that are not well sorted (difference 2 – 3) and the cells that are not counted (difference 3 – 4). The final viability obtained with the system remains close to the real one, most of the difference coming from the sample arriving in the channels already before sorting.

The different sources of errors introduce some limitations on the device, but can be overcome by some adaptation of the design and methods. As can be seen in Fig. 5, the biggest error actually comes from the sampling of the cells from the inlet to the sorting and counting channels, which can be due to occasional sticking of the cells to the chip walls, phenomenon which occurs according to the degree of hydrophobicity of the surface and to its exposed charge. The ability of the miniaturised device to accurately probe the cell sample could be improved by the deposition of a suitable coating on the microchannel wall, such as polyethylene glycol or polyvinylpyrrolidone [22]. Representativeness of the on-chip sample would benefit from homogenisation of the sample at the inlet, which could be done by micro-stirring or dead volume reduction.

The error coming from the wrong sorting of the living and dead cells in the corresponding channels is small. It can come from non-optimised n- and pDEP forces which are used to focus the whole population at one side and to attract living cells to the other one. Moreover, a more pronounced dead cell tendency to sticking can lead to the formation of clusters, whose sorting finally depends on its global properties (size, predominant cell type, etc.). Possible strategies to overcome these intrinsic limitations rely either on the optimisation of the cell concentration and flow rate or on some micro-stirring at the inlets in order to break these clusters apart.

Finally, counting is the third source of error in determining sample viability. Due to the structure of the coulter counter, two or more cells passing at the same time are likely detected as a single, bigger one. Algorithms can be implemented, in order to detect abnormal shapes in current pulses which are due to coincidences, but the

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easiest and most reliable way of preventing these counting errors requires an optimisation of the process parameters (cell concentration, flow rate) or the use of more sophisticated configurations, such as hydrodynamic focusing.

In this work the cells were flowing at around 500 μm/s, giving a flow rate around 0.2 nl/s, and the cells have been sorted and counted at a rate of up to 10 cells per second. As discussed in [2], as long as the cells stay during a sufficient time in the sorting region to reach an equilibrium position, the cell sorting works independently of the flow speed, so that increasing the length of the sorting region can increase the throughput of the device to obtain faster, statistical measurements. Cell counting can also be performed as higher speed, with a maximal operation speed reported at 20,000 cells per minute [23].

4. Conclusions

This paper described the design and fabrication of a chip capable of both electrical cell sorting and counting using the combination of a dielectrophoretic separation and coulter counting. This device was used to evaluate the viability of a mixed sample of living and dead yeast cells. The advantages of such a system are the use of a label-free realtime analysis which is not modifying the sample, and the relatively simple experimental setup without optical components. Moreover, the sorting technique used allows a more subtle separation than the traditional size-based methods, enabling differentiation of cells depending on their dielectric properties. Optimisation of the system can improve the cell sampling in the inlet and diminish the number of clusters flowing in the microchannels, thereby increasing the reliability of the viability measurement. As the sorting and counting can be performed in a fast and automatic way, this lab-on-chip is particularly adapted for point-of-care applications such as diagnosis of insufficient cell concentrations, identification of cell deficiencies or bacterial contamination.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 227243. Thanks to the CMI for the access to the cleanroom facilities.

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Biographies

Guillaume Mernier was born in Libramont, Belgium, in 1981. He obtained his degree of Engineer in Physics in 2003 at the *University of Liège*, with greatest honors. He started a PhD at the *Catholic University of Leuven* and the *Interuniversity Micro-Electronics Center* (*IMEC*) in 2004 on the "Chemical stimulation of neurons by local and controlled release of neurotransmitter", and received his PhD in 2009. Since February 2009, Guillaume occupies a postdoctoral position at the *Swiss Federal Institute of Technology of Lausanne* (*EPFL*), where he works on several topics related to lab-on-chip research.

Niccolò Piacentini was born in Torino, Italy, in 1982. He received the Italian *Laurea Magistrale* degree in biomedical engineering from *Politecnico di Torino*, Torino, Italy and from *Politecnico di Milano*, Milano, Italy, in 2006 and 2007, respectively. Currently he is Doctoral Candidate in biomedical engineering at the *Politecnico di Torino*, and he has recently been an Exchange Student in the *Laboratoire de Microsystèmes* LMIS4, Swiss Federal Institute of Technology (EPFL), Lausanne. His research interests include microsystem technology applied to bioengineering, biomedical signal processing and instrumentation.

Raphaël Tornay was born in Monthey, Switzerland. He obtained his degree of microengineering in 2005 at the Ecole Polytechnique de Lausanne. After an internship at the University of Southampton where he developed a microfluidic system for Raman spectroscopy, he joined the *Laboratoire de Microsystèmes* LMIS4 at EPFL where he obtained his PhD with a thesis titled "Dielectrophoresis-based Continuous-Flow Particle Microreactor". Since July 2009 he works for Biocartis, a company based at the Science Parc of EPFL.

Nina Buffi was born in Locarno, Switzerland, in 1985. She obtained her Master's degree in biomedical microengineering in March 2009 at the Swiss Federal Institute of Technology of Lausanne (EPFL). She did her Master's thesis on "On chip impedance spectroscopy flow cytometry" at the *University of British Columbia of Vancouver*. She started a PhD at the *Swiss Federal Institute of Technology of Lausanne* in May 2009.

Philippe Renaud received his PhD degree in physics from the University of Lausanne, Switzerland in 1988. He was postdoctoral researcher at University of California in Berkeley, USA to develop scanning tunneling microscopes for low-temperature and at the IBM Zürich Research Laboratory where he performed measurements of the local STM induced luminescence. He then joined the Sensors and Actuators group of the Swiss Center for Electronics and Microtechnology (CSEM) at Neuchâtel where he was involved in the design and the technology of microsensors and micro-mirrors for optical switching. In 1993, he joined EPFL as assistant professor but remained part-time collaborator of CSEM until the end of 1994. In summer 1996, he went to Japan as visiting professor at the Tohoku University. In 1997, he was appointed as full professor at EPFL. His research interests are microfluidics and BioMEMS applications.

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