Pumping of mammalian cells with a nozzle-diffuser micropump†

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We discuss the successful transport of jurkat cells and 5D10 hybridoma cells using a reciprocating micropump with nozzle-diffuser elements. The effect of the pumping action on cell viability and proliferation, as well as on the damaging of cellular membranes is quantified using four types of well-established biological tests: a trypan blue solution, the tetrazolium salt WST-1 reagent, the LDH cytotoxicity assay and the calcium imaging ATP test. The high viability levels obtained after pumping, even for the most sensitive cells (5D10), indicate that a micropump with nozzle-diffuser elements can be very appropriate for handling living cells in cell-on-a-chip applications.

Introduction

The analysis of complex biological systems such as living cells using microfabricated structures has attracted much attention in the past few years and the field of 'cellomics' clearly is of growing importance.[1] For example, the effect of drugs or external electrical stimuli on cell behaviour can now be easily tested in microfluidic systems. Analytical standard operations on-chip, using reduced quantities of cells or even single cells, have become feasible[2] and chip-based patch clamping of a single cell has the potential to replace traditional patch electrodes for cellular membrane analysis.[3] Various methods have been proposed to handle minute quantities of liquids containing cells. For example, one has adapted commercially available ink-jet print heads into 'cell printers' for the purpose of positioning viable cells onto pre-defined patterns.[4,5] Ellson performed acoustic droplet ejection of mammalian cells, a method which was shown to be as gentle as a conventional pipette.[6] Santesson *et al.* performed ultrasonic levitation of droplets containing a dozen cells which behaved similarly to non-exposed cells.[7] For many applications, the successful transport and handling of cells on a microfluidic chip[8] requires a safe and dedicated pump[9] that can be integrated with the chip or separated from the sample flow path.

The use of nozzle-diffuser elements in a miniaturized diaphragm pump was presented for the first time by Stemme and Stemme in 1993.[10] Olsson further demonstrated that these elements could be employed in a very attractive way to fabricate simple "valve-less" reciprocating micropumps.[11] The major advantages of nozzle-diffuser elements are their simple geometry together with the absence of moving parts, unlike mechanical check valves.[12,13] The latter aspect suggests that nozzle-diffuser micropumps could be very suitable for the transportation of cells in lab-on-a-chip systems, as indicated by the first experiments of Andersson *et al.*[14] Although successful transport of living cells was reported by these authors, no data were provided on the viability of the cells after pumping.

In this paper, we present a detailed study of the impact of the pumping action on various types of 10 μm size cells. We use an electromagnetically actuated nozzle-diffuser micropump, the fabrication method and characterization of which is detailed in ref. 15. Viability and proliferation, as well as cellular membrane damaging of jurkat cells and 5D10 hybridoma cells are quantified using a trypan blue solution, the tetrazolium salt WST-1 reagent, and the LDH cytotoxicity assay. Also physiological Ca imaging experiments are performed by visualizing jurkat cell responses to adenosine triphosphate (ATP). The high viability levels obtained after pumping suggest the excellent potential of this type of micropump for cellomics. We compare our results with those obtained with a classical peristaltic pump (REGLO Digital MS-4/8, ISM834A V2.10, Ismatec, Glattbrugg, Switzerland). This pump was chosen for its comparable performance with our micropump in terms of achievable flow rate.

Nozzle-diffuser micropump

Nozzle-diffuser elements are fluidic channel constrictions that modify the fluid dynamics such that the fluidic resistance is higher in one direction than in the other, causing the flow rate to be different in the two directions for the same applied pressure. A diffuser is characterized by a gradual widening of the fluidic cross-section in the sense of the flow and a smaller fluidic resistance. A nozzle is characterized by a gradual reduction of the fluidic cross-section in the sense of the flow and a higher fluidic resistance.

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† Electronic Supplementary Information (ESI) available: a video showing the fluid circulation in a nozzle-diffuser micropump and demonstrating its bubble-tolerance is shown. The experiment was done with a 6 Hz and 100 mA sinusoidal excitation of the electromagnet. See http://dx.doi.org/10.1039/b504468e

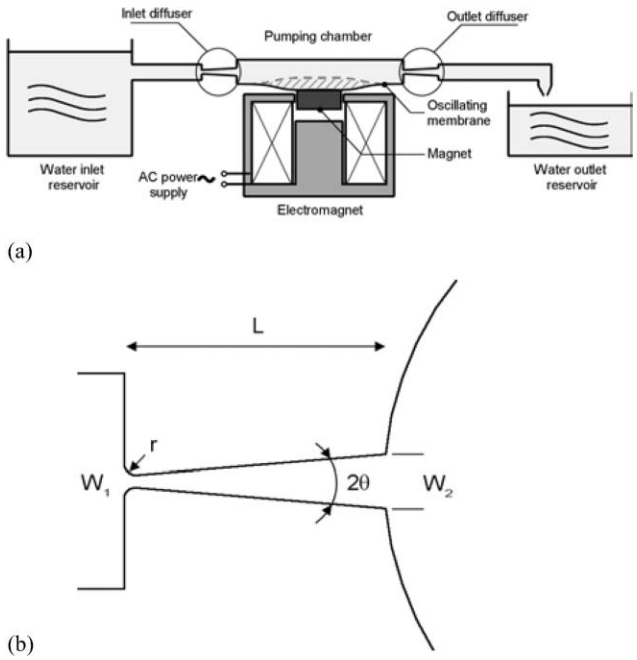

Fig. 1 **(a)** Schematic diagram showing the working principle of the nozzle-diffuser micropump actuated with an external electromagnet. **(b)** Geometrical parameters of the diffuser element.

In the concept of the diffuser-based reciprocating micropump, the pumping effect is obtained thanks to the oscillatory motion of a diaphragm together with the directional effect of two nozzle-diffuser elements (Fig. 1a). The poly-dimethylsiloxane (PDMS) membrane with embedded NdFeB magnet is actuated using an external electromagnet fed by a sinusoidal current. Using a PDMS membrane with high deflection amplitude, we obtain a high compression ratio, as determined by the ratio of the stroke volume (illustrated by the dashed area in Fig. 1a) and the dead volume of the chamber. The high compression ratio renders our pump self-priming and bubble-tolerant† and is a key advantage offered by the use of a silicone elastomer and long range magnetic actuation forces.

The nozzle-diffuser elements are in a reverse orientation relative to the pumping chamber and behave alternately as a diffuser and as a nozzle during each half of an oscillation cycle.[10,11] During the supply mode, the chamber volume increases (Fig. 2a) and more fluid flows through the inlet element than through the outlet element. During the expulsion mode, the chamber volume decreases (Fig. 2b) and more fluid flows through the outlet element than through the inlet element. Vortex-like flow behaviour develops in the chamber during pumping. The pictures of Fig. 2 are taken using a CCD-camera and a fluorescein solution in water (Fluka, Buchs, Switzerland).

Table 1 Water flow rate of the nozzle-diffuser micropump measured for the combination of three sinusoidal excitation frequencies and three current amplitudes of the electromagnet[a]

	10 Hz	50 Hz/100 Hz
50 mA	0.3 mL min^{-1}	0.4 mL min^{-1}
75 mA	0.5 mL min^{-1}	0.6 mL min^{-1}
100 mA	0.6 mL min^{-1}	0.8 mL min^{-1}

[a] Uncertainty: $\pm$0.05 mL min^{-1}.

Fig. 1b shows the geometrical parameters of the nozzle-diffuser element we used. The diffuser entrance has rounded corners (curvature radius $r = 100$ μm) and a width $w_1 = 100$ μm; the outlet has sharp corners and a width $w_2 = 500$ μm. The diffuser length is $L = 2.3$ mm, defining the angle $2\theta = 9.5°$. The height of the nozzle-diffuser element is $h = 300$ μm, as determined by the thickness of the glass sheet out of which it is microfabricated. The pumping chamber diameter is 7 mm and its depth is 0.6 mm (thickness of two glass layers).

A detailed description of the microfabrication of the micropump, as well as its characterization (in terms of water flow rate and back-pressure) can be found in ref. 15. For the specific frequencies and amplitudes of the electromagnetic actuation employed in the experiments described below, the time-averaged flow rates are typically in the range of 0.5–1 mL min^{-1} (see Table 1).

Experimental

Cell culture

The influence of cell pumping on cell viability is tested by using the mouse hybridoma cell line 5D10, and the human T leukaemia cell line jurkat (ATCC TIB152). Cells are cultivated in 75 cm^2 culture flasks using RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 1 mM

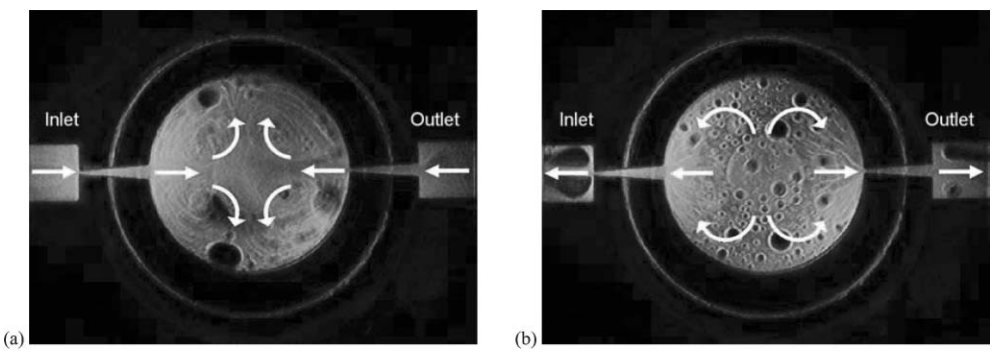

Fig. 2 Photographs of the nozzle-diffuser micropump during different parts of the pumping of a fluorescein solution using a sinusoidal excitation of the electromagnet (6 Hz): **(a)** supply mode and **(b)** expulsion mode. Arrows indicate the sense of the flow and the presence of air bubbles enables a clear visualization of the flow patterns.†

sodium pyruvate, 1% non-essential amino acids, 1% 4-(2-hydroxyethyl)-monosodium salt (HEPES) (all from Gibco® Cell Culture, Invitrogen, Basel, Switzerland). Cultures are maintained at 37 °C under 5% CO_2 and 95% air atmosphere. The growth medium is changed every other day until the time of use of the cells. Cell density and viability, defined as the ratio of the number of viable cells over the total number of cells, of the cultures are determined by trypan blue staining and a Neubauer improved hemacytometer (Blau Brand, Wertheim, Germany). Prior to each viability and cytotoxicity test, the cells are harvested and diluted at a density of 2×10^4 cells mL^{-1} in the WST-1 viability assays and 1×10^5 cells mL^{-1} in the LDH cytotoxicity assays. The cell suspension is seeded into 96-well plates at 200 μL $well^{-1}$, and incubated for approximately 48 hours before WST-1 tests in order to reach confluency.

Viability and cytotoxicity tests

Trypan blue staining of cells is known to be only a qualitative test for monitoring of the cell culture. To quantify the influence of the cell pumping on the viability of the cells, two precise colorimetric viability and cytotoxicity methods are used: the WST-1 test and the LDH test. These tests are performed with the nozzle-diffuser micropump at varying pumping conditions and compared with the results of a peristaltic pump at varying flow rates. The effect of incubation time for cell survival in the WST-1 test is evaluated by performing 1 h, 2 h, 3 h, and 4 h tests. To further evaluate the influence of the cell pumping on the cellular membrane, LDH tests are carried out at 22 °C shortly after manipulation of the cells.

WST-1 test. The WST-1 test (Cell Proliferation Reagent WST-1, Roche, Germany)[16] is performed as follows: cells are seeded in a 96-well tissue culture microplate at a concentration of 4×10^3 cells $well^{-1}$ in 200 μL culture medium and incubated for 48 hours at 37 °C and 5% CO_2. After the pumping experiments, 20 μL WST-1 reagent is added to each well and the plate is shaken for 1 min on a microtiter plate shaker (450 rpm). The plates are further incubated at 37 °C and 5% CO_2 and the absorbance of the formazan product is measured at 450 nm with an ELISA reader (Spectra MAX 340, Molecular Devices, Sunnyvale, CA, USA). Cell viability is calculated from the absorbance values as:

$$\text{viability}_{\text{WST}}\,[\%] = \frac{(A_{450\text{ nm}} - A_{650\text{ nm}})_{\text{exp}}}{(A_{450\text{ nm}} - A_{650\text{ nm}})_{\text{unpumped}}} \times 100 \qquad (1)$$

with $A_{450\text{ nm}} - A_{650\text{ nm}}$ the absorbance difference between 450 nm and 650 nm for the cells that are pumped ("exp") and unpumped. The results are expressed as an average over 6 nominally identical measurements.

LDH test. LDH leakage is measured from jurkat cells and 5D10 hybridoma cells by using the CytoTox 96® Non-Radioactive Cytotoxicity Assay-kit (Promega Corporation, Madison, WI, USA).[17,18] Briefly, after pumping experiments, 100 μL samples are seeded in 96-well plates and 100 μL of substrate mix solution are added. The plates are incubated at 22 °C for 10 min. The LDH that has been released from the cells catalyses the conversion of resazurin into resorufin. After incubation, the enzymatic reaction is stopped by adding 50 μL stop solution (1 M acetic acid) to the wells. Fluorescence values are measured with an excitation wavelength of 485 nm and an emission wavelength of 590 nm in a microplate fluorometer (Cytofluor® series 4000, Perceptive Biosystems, Framingham, MA, USA). The results are expressed as an average over 6 nominally identical measurements. Maximum LDH release after cell lysis using 9% v/v Triton®X-100 and spontaneously released LDH in unpumped cells are also measured. The average fluorescence values of the culture medium background are subtracted from all fluorescence values of experimental wells. Cytotoxicity caused by the pumping is calculated as follows:

$$\text{cytotoxicity}_{\text{LDH}}\,[\%] = \frac{I_{\text{exp}} - I_{\text{unpumped}}}{I_{\text{exp, max}} - I_{\text{unpumped}}} \times 100 \qquad (2)$$

I_{exp} is the fluorescence value of the cells that are pumped, I_{unpumped} is the fluorescence value for the spontaneous release of LDH by unpumped cells and $I_{\text{exp, max}}$ is the fluorescence value for lysed cells with maximum LDH release.

ATP test. Extracellular ATP has been shown to induce significant functional changes in a wide variety of cells and it is used as a control substance to test cell viability.[19,20] Ca^{2+} imaging experiments are performed to visualize jurkat cell responses to ATP. For Ca^{2+} signaling tests, jurkat cells are grown on sterile microscope coverslips precoated with poly-D-lysine. 24 h after seeding, cells are loaded with Ca^{2+}-sensitive fluorescent dye Fluo-3 (Molecular Probes, Invitrogen, USA) by bathing in serum-free medium containing dye for 30 min at 37 °C. Thereafter, Fluo-3 containing medium is replaced by medium supplemented with 10% fetal calf serum and, as before, incubated for 30 min at 37 °C. Subsequently, dye-loaded cells are washed with PBS buffer and subjected to Ca^{2+} imaging. 1 mM ATP solution is applied to the cells, and the time-dependent fluorescence is measured with an excitation wavelength of 488 nm and emission wavelength of 510 nm using a Zeiss LSM 510 microscope (Carl Zeiss AG, Germany).

Results

Cell viability and cytotoxicity

The jurkat cells can be successfully pumped with the nozzle-diffuser micropump, as shown in Fig. 3. Fig. 3a represents naturally clustered jurkat cells in a culture medium at 37 °C and Fig. 3b shows these cells separated after passage through the micropump. Fig. 3c is the picture of the cells 24 h after pumping. No significant loss of viability is associated with the disruption of the cell clusters, as measured with trypan blue (viability of 98% for the cells shown in Fig. 3b and 99% for the cells shown in Fig. 3c). This is further confirmed by the observation that after 24 h of incubation of the cells, new clusters are formed and the cell density increases (cell density of 4×10^5 cells mL^{-1} in Fig. 3b and 1×10^6 cells mL^{-1} in Fig. 3c).

Table 2 shows the viabilities of the cells pumped with the micropump and the peristaltic pump under different operating

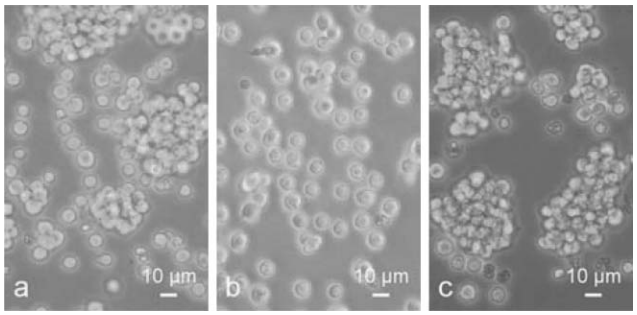

Fig. 3 Typical observation of jurkat cells **(a)** in a culture medium at 37 °C and **(b)** just after their passage in the diffuser micropump (actuation at 100 Hz, 75 mA) and **(c)** after 24 hours.

Table 2 Cell viability values (%) using the WST-1 test after pumping after 4 h incubation

Pumping conditions	Jurkat	5D10
Micropump (75 mA, 10 Hz)	73 ± 11	70 ± 15
Micropump (75 mA, 50 Hz)	80 ± 1	120 ± 40
Micropump (75 mA, 100 Hz)	77 ± 7	110 ± 30
Peristaltic (0.5 mL min^{-1})	94 ± 12	73 ± 14
Peristaltic (1 mL min^{-1})	80 ± 14	68 ± 10

conditions, as measured by the WST-1 test. Compared to the unpumped cell population, we find a viability of around 80% for the jurkat cells with a relatively small standard deviation and around 70–100% for the 5D10 cells, though with a much higher standard deviation. The relatively large variability of the latter result is consistent with the fact that 5D10 mouse hybridoma cells are known to be very sensitive to shearing, which can induce substantial loss of their viability after pumping. Except for the variation in results for the 5D10 cells, no clear difference between the micropump and the peristaltic pump is observed with regard to cell viability.

Table 3 Cell cytotoxicity values using the LDH test[a] after pumping, for different pumping conditions

Pumping conditions	Jurkat	5D10
Micropump (75 mA, 10 Hz)	2.6 ± 0.9	14 ± 3
Micropump (75 mA, 50 Hz)	4.6 ± 1.1	12 ± 6
Micropump (75 mA, 100 Hz)	7.1 ± 0.9	15 ± 3
Peristaltic (0.5 mL min^{-1})	4.6 ± 1.2	14 ± 3
Peristaltic (1 mL min^{-1})	2.7 ± 1.6	14 ± 1

[a] At 22 °C and after 10 min incubation.

Table 3 evaluates the influence of pumping on cellular membrane damage using the LDH assay. The measured LDH leakage 10 min after pumping for jurkat cells is between 3 and 7%, indicating no major damage on the cellular membrane. The LDH cytotoxicity values for the delicate 5D10 cells are higher, around 15%. This is consistent with the viability data of Table 2 and could indicate a certain cell lysis of these cells during pumping.

Effect of pumping parameters on cell viability

Fig. 4 shows the effect of different coil actuation currents (75 mA and 100 mA) on cell viability, as determined by the WST-1 test. Fig. 4a and 4b show the jurkat cell viability indicating that for the range of frequencies and current used,

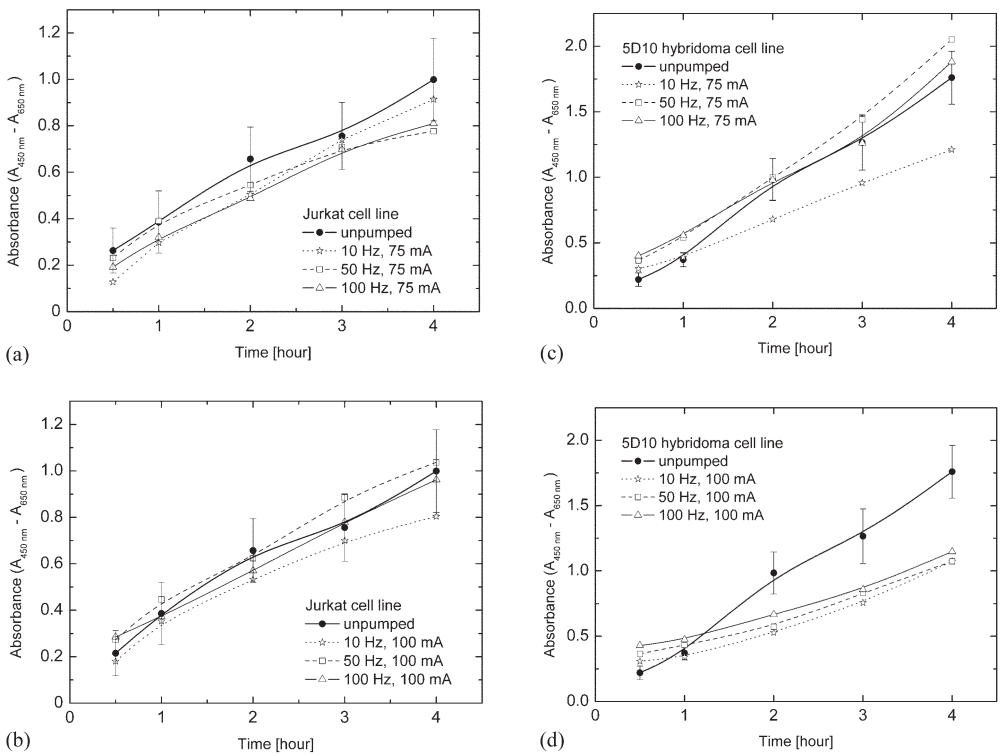

Fig. 4 WST-1 viability test of **(a,b)** jurkat cells and **(c,d)** 5D10 hybridoma cells for different pumping conditions. Each point represents the average over 6 nominally identical measurements. For clarity, the standard deviation is only represented for the unpumped data, but is of the same order for the other curves.

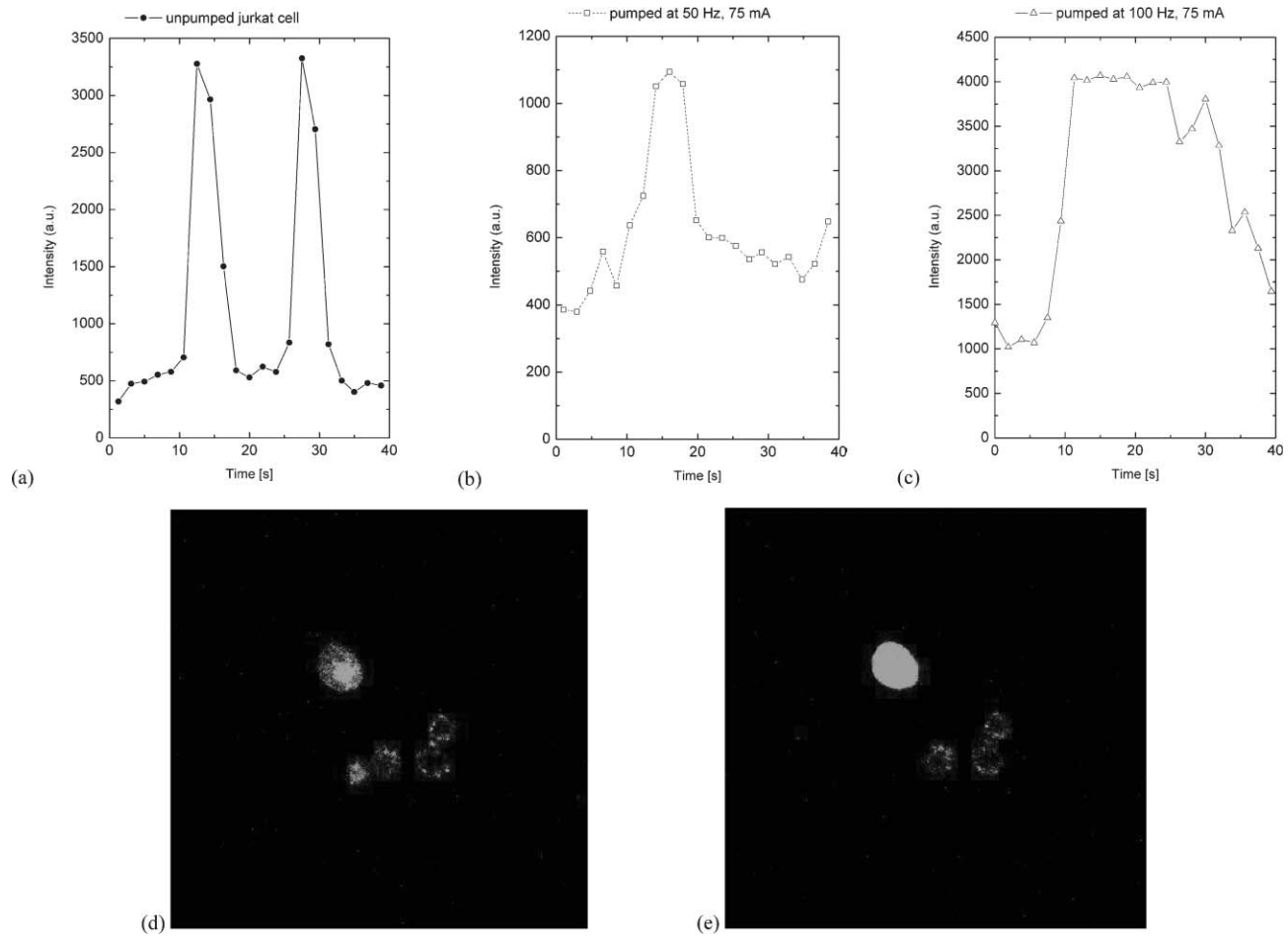

Fig. 5 ATP viability test of **(a)** unpumped jurkat cells and **(b,c)** cells pumped with the micropump using an actuation current of 75 mA, **(b)** at 50 Hz and **(c)** at 100 Hz. **(d,e)** Confocal micrographs showing a single jurkat cell **(d)** before and **(e)** during ATP stimulation.

there is no significant change in cell viability as compared to the unpumped culture. Fig. 4c and 4d show the same measurements but for the 5D10 cells. Fig. 4c shows that only actuation at 10 Hz significantly affects the viability of cells while the 50 Hz and 100 Hz are similar to the unpumped solution. We attribute this to the longer residence time in the pump together with the large amplitude of the oscillations for this range of frequencies. When pumping with a coil actuation current amplitude of 100 mA, we see that for all frequencies used, the cell viability is below that of the unpumped solution (Fig. 4d).

As indicated in Table 1, a higher actuation current results in an enhanced flow rate, so that cells are increasingly subjected to shear forces and cell lysis. Summarizing our results, we have proven the successful pumping of jurkat and 5D10 hybridoma cells. Viability is around 80% for both types of cell solutions with a relatively small standard deviation for the jurkat cells and a larger variation for the less 'robust' 5D10 cells.

Calcium imaging

Jurkat cell viability has also been analyzed by measuring cell responses to ATP exposure. From previous work, it is known that ATP can evoke specific calcium signaling in a wide variety of cells. ATP activates P2-purinergic receptors present in the membrane, which stimulates IP_3 formation, followed by a transient increase of intracellular Ca^{2+} in the cytosol. The transient fluorescent intensity graphs of Fig. 5(a–c) show that the ATP application effectively induces the Ca^{2+} increase in the unpumped cells as well as in cells that are pumped using two different conditions. Fig. 5d and 5e represent confocal micrographs of the fluorescent response of a single jurkat cell before and during ATP stimulation, respectively. Our results indicate again that the pumping does not affect cell viability.

Conclusion

We have used an electromagnetically actuated nozzle-diffuser micropump to study the viability and cytotoxicity of jurkat cells and 5D10 hybridoma cells. The tetrazolium salt WST-1 reagent and the LDH cytotoxicity assay were used to systematically study the cell behaviour as a function of pumping parameters (actuation current and frequency). Viability test of jurkat cells was further confirmed by the calcium imaging ATP test. The high viability levels (in the 80% range, even for the most sensitive cells) obtained after pumping suggest the excellent potential of this type of micropump for cellomics.

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