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● *Original Contribution*

PROLIFERATION AND VIABILITY OF ADHERENT CELLS MANIPULATED BY STANDING-WAVE ULTRASOUND IN A MICROFLUIDIC CHIP

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Abstract—Ultrasonic-standing-wave (USW) technology has potential to become a standard method for gentle and contactless cell handling in microfluidic chips. We investigate the viability of adherent cells exposed to USWs by studying the proliferation rate of recultured cells following ultrasonic trapping and aggregation of low cell numbers in a microfluidic chip. The cells form 2-D aggregates inside the chip and the aggregates are held against a continuous flow of cell culture medium perpendicular to the propagation direction of the standing wave. No deviations in the doubling time from expected values (24 to 48 h) were observed for COS-7 cells held in the trap at acoustic pressure amplitudes up to 0.85 MPa and for times ranging between 30 and 75 min. Thus, the results demonstrate the potential of ultrasonic standing waves as a tool for gentle manipulation of low cell numbers in microfluidic systems. (E-mail: jessica.hultstrom@biox.kth.se) © 2006 World Federation for Ultrasound in Medicine & Biology.

INTRODUCTION

Contactless handling and manipulation of cells in microsystems are important for the development of automated and efficient cell-based biotechnology applications. In applications that utilize delicate cells, it is important to avoid unwanted physical surface contact as well as interference with any biologic process caused by the manipulation tool. Ultrasonic-standing-wave (USW) technology shows promise for both efficient, as well as gentle, manipulation of cells. Here, we investigate the cell viability by studying the proliferation rate of adherent COS-7 cells after ultrasound exposure in a microchip-based USW trap.

Reported methods for contactless manipulation of individual cells in microchips are most often based on laser tweezers (Enger et al. 2004) or dielectrophoresis (Müller et al. 2003). USW technology is an interesting alternative that has been introduced to microchips, *e.g.*, for continuous cell separation (Harris et al. 2003, Petersson et al. 2004, Kapishnikov et al. 2006), cell washing (Hawkes et al. 2004a, Petersson et al. 2005) cell deposition on a surface (Hawkes et al. 2004b) and cell positioning (Haake et al. 2005). Common for those approaches is the use of MHz-frequency ultrasound in microchips for manipulation of large groups of cells during short terms. However, in contrast to optical tweezers and dielectrophoresis, USWs have also been shown to be very suitable for long-term manipulation. This has been demonstrated in macroscaled systems, *e.g.*, for cell retention and filtering in high-density perfusion processes (Shirgaonkar et al. 2004). Here, ~1000 h of operation is typically carried out with no significant loss in cell viability. However, to minimize the cell damage, several parameters must be carefully controlled, *e.g.*, the acoustic pressure level, the flow properties and the temperature. Therefore, it is of interest to investigate whether the results from high-density cell samples in macrosystems also are applicable to low-density cell samples in microchips.

In macroscaled systems (*i.e.*, with cm- or mm-scaled resonators), the viability of USW-manipulated cells has been measured by different methods. Most often, it is measured directly in connection with the exposure. Typically, the measured parameter is the integrity of the cell membrane, which is determined by, *e.g.*, the use of trypan blue dye or propidium iodide (Kilburn et al. 1989; Doblhoff-Dier et al. 1994; Pui et al. 1995; Wang et al. 2004; Bazou et al. 2005a; Khanna et

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al. 2006). Another similar strategy is to measure the release of intracellular components such as potassium ions or haemoglobin from red blood cells (Yasuda 2000; Cousins et al. 2000). Furthermore, early and late apoptosis have been measured by the use of fluorescence assays (Bazou et al. 2005a). In perfusion applications, the viability has been measured indirectly by studying the production rate of proteins, monoclonal antibodies or viruses (Zhang et al. 1998). In addition, transmission electron microscopy (TEM) has been used for detailed examination of the structure and morphology of intracellular components (Kobori et al. 1995; Radel et al. 2000). Besides, physical variables such as fluid flow, temperature and possible cavitation around ultrasonically trapped cells have been thoroughly investigated (Bazou et al. 2005b). In all these macroscale studies the cell viability, directly after exposure, is typically 95 to 99% under controlled conditions (*i.e.*, at moderate pressure levels).

Another, less common, approach to measure the cell viability is to investigate the proliferation rate of recultured cells after ultrasonic exposure. This method should be a more sensitive tool for quantification of viability, since it also takes into account any possible effects that may cause delayed damage to the cells. This hypothesis is supported by studies of the physical factors involved in ultrasound-mediated damage of cells in standing-wave systems. For example, one study suggests that the structure of the cytoskeletal elements responsible for the cell division process (*e.g.*, spindle bodies and microtubules) may be partially damaged or passivated by mechanical stress from ultrasound traps (Pui et al. 1995). Furthermore, ultrasonic exposure may alter the integrity of the cell vacuole in trapped yeast cells (Radel et al. 2000). Thus, proliferation is indeed an interesting parameter for sensitive viability investigation. Reports on cell proliferation after standing-wave ultrasonic exposure are, *e.g.*, investigation of the proliferation of yeast cells fixed in a semirigid nontoxic gel matrix (Gherardini et al. 2005) and investigation of the proliferation of hybridoma cells exposed to different ultrasound energies (Pui et al. 1995). However, both these studies have only investigated proliferation after short-term ultrasonic exposure (<10 min).

In the present paper, we study the proliferation rate of recultured adherent cells after both short- and long-term ultrasonic standing wave exposure in a microfluidic chip. In contrast to the long-term studies in macroscaled perfusion system with high cell densities, we investigate the viability after individual handling of low cell numbers ($\sim 10^2$–10^3) and low cell densities ($\sim 10^4$ mL^{-1}) in microsystems. Thus, other effects than ultrasound that may cause stress or damage are also included, *e.g.*, fluidic shear forces from passages through narrow microchannels and syringe needles and increased rate of surface contact in the high surface-to-volume microchannels. In addition, we study the effect on the proliferation rate of cells concentrated in 2-D aggregates, compared with nonconcentrated cells. The fundamental motivation of this work is to investigate the applicability of chip-based USW tools for gentle and long-term handling of individual cells within a wide range of biotechnology applications.

MATERIALS AND METHODS

Ultrasonic microfluidic chip assembly

The ultrasonic standing wave microfluidic chip was fabricated in house. The chip assembly is illustrated in Fig. 1. A 260 μm thick polydimethylsiloxane (PDMS) layer (Sylgard 184, Dow Corning, Midland, USA) was used as spacer between the two glass plates. The depth of the microchannel was close to half the ultrasonic wavelength in water ($\sim$250 μm) resulting in one pressure node in the middle of the channel. The 550 μm thick glass plates (21 $\times$ 23 mm borosilicate cover glass, Mentzel GmbH, Braunschweig, Germany), which defined the acoustic resonator, worked both as coupling layer and quarter-wavelength reflectors. A 3-MHz circular 5-mm diameter lead zirconate titanate (PZT) transducer (PZ26, Ferroperm, Kvistgaard, Denmark) was attached by conductive glue (Thermoset MD-120, Lord Chemical Products, Manchester, UK) on the top glass plate. The chip was designed with two separate inlets to facilitate rapid washing during cell trapping experiments. On the inlet side, a PDMS block was fabricated and attached to the side of the chip for easier and more stable connection between the chip and the external fluidic system. The latter consisted of Teflon tubing and needles with inner diameters of 250 μm and 110 μm, respectively. On the outlet side, a free-hanging 5 $\times$ 5 mm thin PDMS flap of thickness $\sim$150 μm was added beneath the open end of the channel to allow easy ejection from the channel without dead volumes and sample losses.

Ultrasonic trapping forces

Cells suspended in an aqueous medium are trapped in the pressure nodes of the ultrasonic standing-wave field. Following the formalism derived by Gor'kov (1962) and assuming a plane field propagating along the vertical z-axis, the acoustic radiation force, $F(z)$, is given by

$$F(z) = \frac{\pi}{2\rho_0 c_0^{\,3}}\left(f_1 + \frac{3}{2}f_2\right)\cdot V\cdot p_0^{\,2}\cdot \nu\cdot \sin\left(2\pi\frac{z}{\lambda_0/2}\right), \quad (1)$$

where ν is the frequency, V is the cell volume, p_0 is the pressure amplitude and λ_0 is the acoustic wavelength in the medium. The dimensionless factors $f_1 = 1 - \frac{\rho_0 c_0^{\,2}}{\rho c^2}$ and $f_2 = \frac{2(\rho-\rho_0)}{2\rho+\rho_0}$ depend on the density and sound velocity of the trapping medium (ρ_0 and c_0, respectively) and the

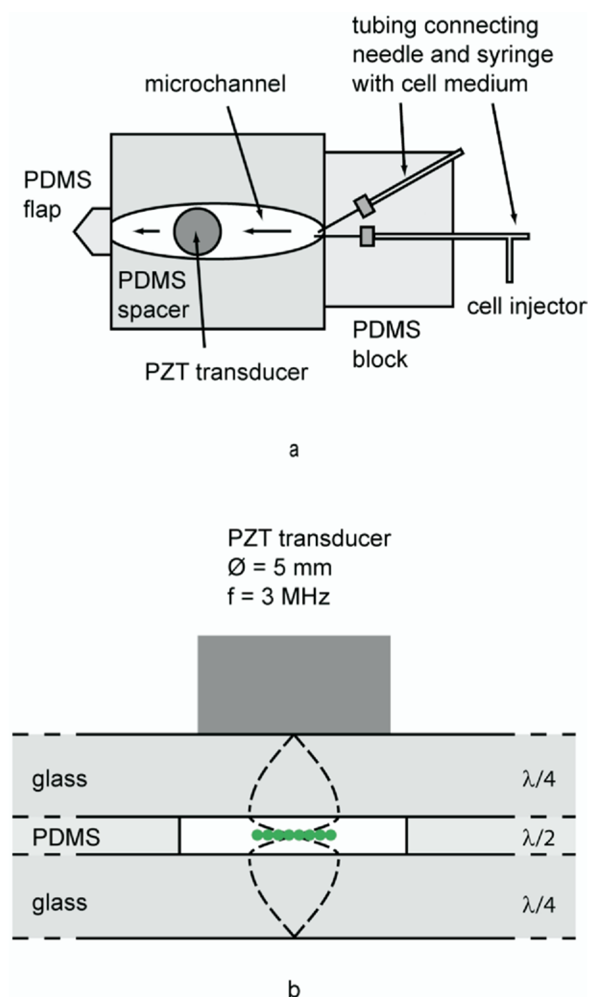

Fig. 1. (a). Top view of the in-house glass-PDMS-glass microfluidic chip with an integrated PDMS block containing two separate inlets with tubing and needles and the outlet with a thin PDMS flap added beneath the open end of the channel. (b). Cross-section of the microfluidic chip showing the circular 3-MHz PZT transducer and the three-layered structure. The channel height of 260 μm, defined by the PDMS spacer, was close to half the ultrasonic wavelength in water, giving one pressure node in the middle of the channel, where the cells were trapped. The glass plates had a thickness of 550 μm, corresponding approximately to a quarter wavelength for the sound in glass.

cell (ρ and c). Initially, the cells move within seconds along the z-axis, due to the strong axial component of the acoustic radiation force. Once trapped in a nodal plane, the cells form 2-D aggregates due to the weaker lateral component of the acoustic radiation force and the attractive interaction force (Bjerknes force) working only at short distances ($\sim\mu$m) (Wiklund and Hertz 2006). There are also other forces acting on the cells that influence the trapping performance, such as the viscous drag force from the flow. In our system (cf. Fig. 1), this drag force is balanced by the weaker lateral components of the acoustic primary force, which is about 100 times lower than the axial force. Hence, a relatively high acoustic pressure amplitude is required for stable trapping against the flow. In addition, at higher pressures, acoustic streaming may significantly influence the stability of the trapped cell aggregates (Kuznetsova and Coakley 2004).

Acoustic pressure level inside chip

To estimate the acoustic pressure amplitude within the microchannel, a gravitational escape experiment was performed. A single 10 μm green-fluorescent latex bead (Bangs Laboratories, Fischer, USA) was trapped at a transducer driving voltage of 0.2 V (peak-to-peak) balancing against the gravity force, $F_g = (\rho - \rho_0)Vg$. This equilibrium can be used to estimate the absolute magnitude of the acoustic trapping force, eqn 1, and thereafter the acoustic pressure amplitude, p_0. The estimated pressure amplitude in the microchannel was 0.57 to 0.85 MPa at a transducer driving voltage of 6 V, depending on the exact location below the transducer.

Chip preparation

Before performing any experiment, the chip and all other reused parts were sterilized using 70% ethanol. The chip was then mounted on a plastic holder and placed in the inverted epifluorescence microscope (Axiovert 135M, Zeiss, Germany) equipped with 10×/0.25NA objective, a CCD-camera and suitable fluorescence filters. The fluidic system, consisting of Teflon tubing, needles, adaptors and glass syringes, were assembled. The laminar flow rate was controlled by a syringe pump (SP2101WZ, World Precision Instrument, Sarasota, USA). The cell injector (Cytocon Injector, Evotec Technologies GmbH, Hamburg, Germany) was placed in close vicinity to the chip, to minimize the tubing distance. Before each experiment, a new sterile PDMS flap was attached at the open end of the channel. A continuous flow of cell medium (Dulbecco's modified eagles medium, Sigma-Aldrich, Stockholm, Sweden) was started at a flow rate of 5 μL/min ($\sim$50 μm/s). The output from the chip was collected in the eight-well cell culture dish (Lab-Tek 8 chamber, Nunc, Rochester, USA) mounted on the chip holder in the microscope. The microfluidic chip and cell culture dish were kept at a constant temperature of 37°C inside a sealed plastic hood connected to warm air incubation by a heating unit with regulated temperature control (Zeiss, Germany). Temperature and flow stabilization after closing the hood normally took about 30 min.

Cell preparation

In parallel with the assembly of the chip, the cell suspension was prepared. The employed adherent COS-7 cells (purchased from ECACC no.87021302), derived

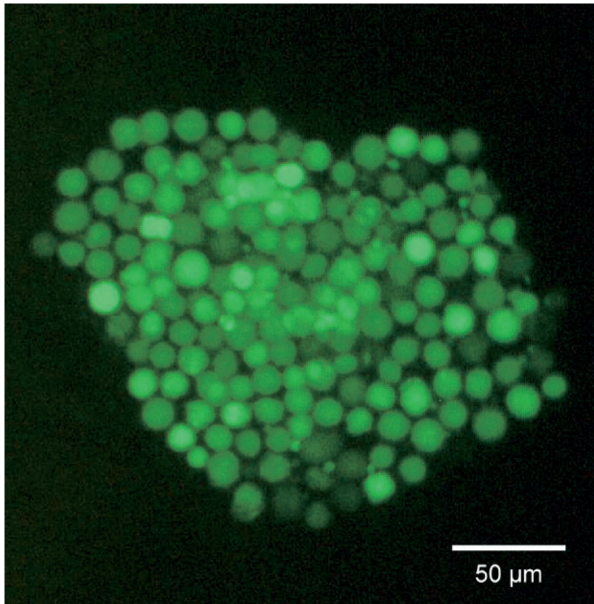

Fig. 2. Image of fluorescently labeled (calcein AM) viable cells trapped by standing wave ultrasound in the microfluidic chip. The cells formed 2-D aggregates stably trapped against the continuous flow of cell medium.

from fetal monkey kidney, were cultured in Dulbecco's modified eagles medium containing 10% fetal bovine serum (Gibco, Invitrogen, Stockholm, Sweden), 1% penicillin streptomycin (Sigma-Aldrich) and 1% L-glutamine (Sigma-Aldrich) and incubated at 37°C in 5% CO_2 atmosphere. The cells were removed from the culture dish by trypsination (0.25% trypsin (trypsin-EDTA, Gibco) for 5 min at 37°C), followed by centrifugation and resuspension in new medium at a typical concentration of 1.5×10^6 cells/mL. For use as a viability indicator and to make the cells visible once inside the microchannel, calcein AM (500 μM) (Molecular Probes, Eugene, OR, USA) was added at a concentration 2 μL per ml of cell suspension, followed by incubation for 30 min in warm water bath (37°C).

Cell trapping experiments

Typically, a 15 to 20 μL sample plug of cell suspension was added via the cell injector (cf. Fig. 1) into the laminar flow of cell medium (flow rate ~5 μL/min). The PZT transducer was operated at a voltage of 6 V (peak-to-peak) and at the channel resonance frequency close to 3 MHz. The cell trapping started about 1 min after injection, when the cells reached the trapping center below the transducer. Here, a few 2-D cell aggregates, as shown in Fig. 2, were formed and stably trapped against the continuous flow. Different USW exposure times, ranging from 30 to 75 min, were investigated with regards to cell viability and proliferation. However, the minimum exposure time was about 5 min, depending both on the volume of the sample plug and on the tubing length between cell injector and chip. Before turning off the ultrasonic field, images were acquired of all fluorescently-labeled cell aggregates, for later counting. The USW-exposed cells were then ejected through the open chip end into a sterile eight-well cell dish by simultaneously turning off the USW and applying manual pressure on the second syringe to increase the flow rate. The thin PDMS flap allowed drops to form and fall down into one of the wells on the cell dish, while still maintaining a constant flow inside the chip. Three drops with trapped cells were collected, giving an initial volume of 30 to 50 μL. The cell dish was then incubated at 37°C and 5% CO_2 (Mini-Galaxy, LabRumKlimat, Stockholm, Sweden) for 10 min, to allow the cell aggregate to sediment. The prepared cell culture medium was added (300 μL) into each well before further incubation for cell cultivation studies. Each experiment was performed two or three times to obtain comparable data points.

Cell counting

Traditionally, cell counting is performed indirectly by estimation of the cell concentration from a small aliquot of the sample (*e.g.*, with a Bürker glass chamber). However, in the present work, we handle low cell numbers (~10^2–10^3) and low concentrations (~10^4 cells/mL). For such cell samples, the Bürker method would cause significant sample losses, resulting in unreliable statistics. Consequently, a direct cell counting method has been used. It is designed to suit our proliferation experiments, assuming exponential cell growth according to

$$N_2 = N_1 e^{at}, \quad (2)$$

where N_1 is the initial cell number and N_2 is the final cell number after a few days of cultivation. These cell data are used for determining the growth factor a. As a result, the cell doubling time, t_{double}, can be calculated

$$t_{double} = \frac{\ln 2}{a}. \quad (3)$$

Following 2 to 3 d of cell cultivation in the incubator the total number of cells was determined from images acquired in a scanning microscope. The procedure included automatic scanning and image acquisition of the whole cell dish area followed by cell counting. Before the microscopy scanning, calcein AM (500 μM) was added to the cell dish (0.5 μL per well) and incubated for 30 min in 37°C. Calcein AM was used primarily as a cell viability indicator (monitoring the esterase activity and membrane integrity), but also for labeling of the cells for the fluorescence microscopy. The cell scanning proce-

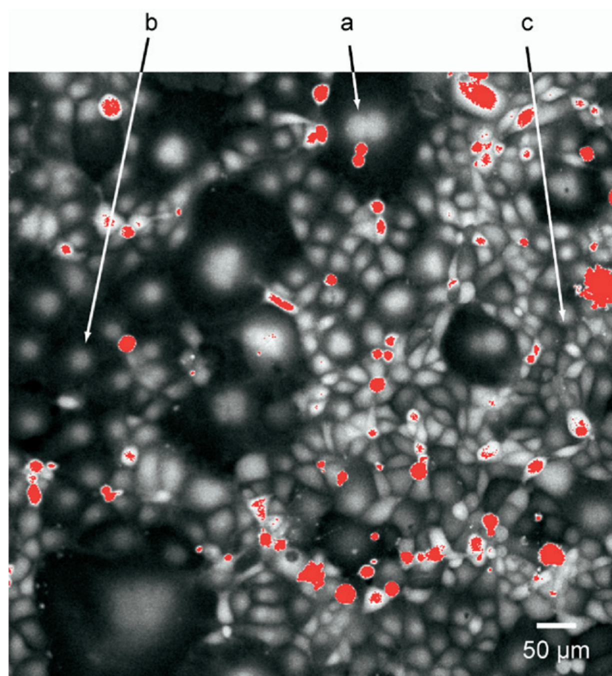

Fig. 3. Confocal microscope image of recultivated COS-7 cells 3 d after USW exposure. Since the cells were labeled with the viability indicator calcein AM, only living cells are visible. To simplify image analysis and cell counting, some high density cell areas must be overexposed and thus saturated. (a). Cell in division phase. (b). Low cell density. (c). High cell density.

dure, performed in a confocal microscope (Zeiss LSM410, Zeiss, Germany), was mainly composed of three steps. First, the cell dish with coverslip bottom (thickness 200 μm) was placed at the microscope stage and the focus and contrast settings were manually adjusted. The next step was to define the center of the specified well on the cell dish by first choosing the shape of the well and then finding the outer edges of the well. Finally, the whole well was automatically scanned, collecting about 200 images (see Fig. 3) from a single well. The acquired images of the cultivated cells were either automatically or manually counted with the free software ImageJ (more information available at http://rsb.info.nih.gov/ij/). For time-saving purposes, an automatic cell counting algorithm has been developed to evaluate the large number of cell images. This method is based on a macroprogram of predefined functions in ImageJ and works on an image sequence containing approximately 200 images originating from a single cell well. The image processing consists of several steps: make and filter binary images, apply watershed and despeckle functions and, finally, count particles. The output from the program gives the final number of cells in the present well after cell cultivation. The automatic counting corresponded well with manual counting (within 10%). However, at very high cell densities, the difference grew to about 30%. Therefore, the presented data (see Results section) relies on manually counted cells, taken as an average of two independent operators.

The initial number of cells was estimated by the same manual counting method, as described above, of trapped cells in the images acquired at the end of each trapping experiment inside the microchip (see Fig. 2). In addition, the ejection step was carefully monitored in a control experiment where the USW trapped cells were counted both before and after ejection. A few (about five) cells attached to either the PDMS flap or the chip during the ejection, but this was accounted for during the cell trapping experiments.

Control experiments

Control experiments without USW exposure were performed with cells subjected to the same cell handling protocol as the USW-trapped cells. The cell suspension was counted in a Bürker chamber and then diluted to give a final concentration of $\sim 10^4$ cells/mL. A small droplet of 10 μL was placed in each of four wells in four cell dishes and the initial numbers of cells were $\sim$1000 cells. The cell dishes were incubated for 10 min and 400 μL culture medium was added to each well. Two of the four cell dishes (eight wells in total) were scanned in the confocal microscope after 1 h, to estimate the average initial cell number. The other two were cultivated in the same incubator as the USW-trapped cells for 3 d before cell counting. This gave the average final cell number for the control cells.

RESULTS AND DISCUSSION

The purpose of our study is to measure the proliferation rate of adherent cells as a function of ultrasonic exposure time in a USW microfluidic chip. In all experiments, the ultrasonic pressure was kept constant at a level approximately twice the minimum pressure amplitude needed for controlled and stable trapping performance. Typically, 500 to 2000 cells were collected and trapped during each exposure and all measurements were done in the same microfluidic chip.

The examination of USW-trapped cells after about 3 d of cultivation showed that the cells survive and proliferate after the microfluidic USW handling. The growth rates were estimated via the cell number doubling times, t_{double}, by comparing the initial and final numbers of cells and assuming exponential growth (cf. eqn 3). In all measurements, only viable cells were counted (by the use of the viability indicator calcein AM). The results are presented in the diagram in Fig. 4. First, six experiments with 30 min exposure time were carried out. Here, cells from two different cell preparations were used. The cells

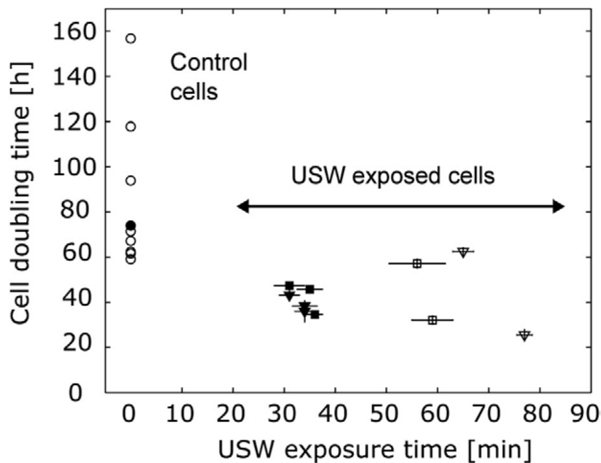

Fig. 4. Cell doubling times were estimated by comparing the initial and final number of cells and assuming exponential growth (cf. eqn 2). Two experiments with a USW exposure time close to 30 min [filled square], [filled inverted triangle] and two experiments for USW exposure times around 60 min were performed [open square], [open inverted triangle]. For comparison, eight batches of untreated cells [open circle] were also cultivated. The average cell doubling time for the control cells is indicated by [filled circle].

were exposed to USWs of average pressure amplitude (0.85 MPa at an applied voltage of 6 V) for about 30 min. Cell samples were collected from each experiment and cultivated. When no effect on cell viability was observed at this exposure time, four experiments were performed at 60 to 75 min. At these longer exposure times, two cell samples could be taken from each cell preparation (for comparable cell handling times). Longer USW exposure times were not possible to measure, due to the lack of nutrient for the cells. However, this could likely be solved by slowly perfusing the trapped cells with cell culture medium containing serum and amino-acids. Repeated measurements for the two different exposure times gave similar doubling times, 35 to 47 h and 25 to 62 h, respectively, and data points from the same cell preparation also showed small variance. The error bars for the cell doubling times depend both on uncertainties in the manual counting of the initial and final numbers of cells as well as on the difference in exposure times for cells being trapped first or last, typically 3 to 11 min.

The experimental data indicate that there exist neither direct nor delayed damaging effects on cells handled and trapped by ultrasonic standing waves (USWs) in a microfluidic system. Furthermore, the proliferation rate of cells exposed to USWs up to 75 min at ~0.85 MPa (cf. Fig. 4) does not deviate from typical values for adherent cells (~24 to 48 h) (Freshney 2000; DSMZ 2006), not even for our samples with low cell numbers ($\sim10^2$–10^3) and low cell concentrations ($\sim10^4$ mL^{-1}). In fact, the untreated control cells (also at concentration $\sim10^4$ mL^{-1}) that were not exposed to ultrasound showed a significantly slower rate of proliferation. Therefore, we may conclude that USW manipulation of cells is not only nondamaging under controlled conditions, but also beneficial for the proliferation rate. We believe that this is due to the increased local cell density obtained when the USW-formed aggregates are transferred from the chip to the bottom of the culture dish. This should be especially important in microchip-based applications, where small and/or diluted cell samples are often employed. Furthermore, additional chip-related factors, besides ultrasound, that may cause damage or stress to the cells do not seem to have any significance. These factors include, *e.g.*, fluidic shear forces and increased surface contact inside narrow microchannels and syringe needles and the light exposure when imaging the ultrasonically-trapped cells before ejection (1 to 5 min). Finally, it should be mentioned that the maximum pressure amplitude level used in this work (~0.85 MPa), is more than sufficient for USW manipulation in a chip. This is especially true, since we here use the much weaker lateral force components of the USW, competing with the viscous flow forces. Thus, even lower acoustic pressure amplitude would be needed for stable cell manipulation if the standing wave direction is parallel with the channel. Typically, the axial USW force component is ~100 times higher than the lateral component in plane-parallel USW resonators (Wiklund and Hertz, 2006). However, we have not measured the upper pressure amplitude limit for nondamaging USW manipulation. The reason is that the trap is not stable under such conditions, due to acoustic streaming. Therefore, such pressure levels are not interesting from an application point-of-view.

The general purpose of the present study is to investigate the applicability of USW technology for gentle cell handling in microchips. Most of the reported biotechnology applications that utilize USW technology handle large sample volumes and high cell concentrations. On the other hand, many applications require handling of individual cells, *e.g.*, for single cell characterization. Therefore, other manipulation tools with higher spatial accuracy are often chosen, *e.g.*, laser tweezers or dielectrophoresis. However, in terms of cell viability, both these methods have been shown to be less suitable for long-term manipulation. An approach to solve this problem is to combine different manipulation technologies, *e.g.*, USW and dielectrophoretic (DEP) manipulation (Wiklund et al. 2006). For example, DEP can be used initially for high-precision manipulation of individual cells, followed by prolonged USW retention of already trapped cells. A suggested application of such a cell handling device is controlled cell differentiation by surface stimulation via artificial immobilization of macromolecules on, *e.g.*, beads. This idea is supported by,

e.g., experiments where cells are differentiated by adhesion to a surface coated with either immobilized cytokines (Leclerc et al. 2006) or immobilized extracellular matrix molecules (Flaim et al. 2005). Here, USW technology can be used for long-term trapping and positioning of a cell-bead complex during the differentiation process (which is assumed to take several hours). Other possible biotechnology applications are live cell assays that require long-term manipulation, or concentration, storage and cultivation of small cell samples (*i.e.*, low cell numbers, small sample volumes and/or low cell concentrations). Furthermore, USW technology can be used for automated cell preparation (*e.g.*, washing and separation) by replacing standard methods based on, *e.g.*, centrifugation and micropipetting.

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