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Cell and particle trapping in microfluidic systems using ultrasonic standing waves

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LUND UNIVERSITY

Doctoral Thesis
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Abstract
Analysis methods are currently being miniaturized in order to save time and money while achieving higher sensitivities. The ultimate goal is to create a lab-on-a-chip where all analysis steps and instruments can be automated and integrated into a single chip. In order to perform cell assays and microparticle based bioassays on chip, methods to manipulate particles and cells in microsystems are desired. This thesis describes the development of a non-contact method of manipulating cells and particles in lab-on-a-chip systems based on ultrasonic standing waves. A short review on microfluidics and acoustics is presented, followed by an overview of other techniques for trapping particles and cells in microsystems. Previous work done within the field of acoustic trapping in macro- and microsystems is reviewed before the development and fabrication of the acoustic trapping platform is presented. The trapping platform provides a non-contact way of immobilizing cells and particles in a continuously flowing microsystem. The possibility to use an array of trapping sites and move particles between different trapping sites is demonstrated. A model bioassay is presented to show the potential of the dynamic arraying concept, where the combination of microfluidics and an array of non-contact trapping sites is used to create a flexible platform for particle-based assays. The platform is also shown to be a gentle way of immobilizing live cells as demonstrated by culturing yeast cells suspended in a standing wave. A viability assay on levitated neural stem cells is also performed to show handling of a more sensitive cell type. The technique is applied to the field of forensics in sample preparation for DNA-analysis in rape cases. The acoustic technique is shown to achieve comparable purities of the separated DNA fraction in a substantially shorter time as compared to the standard methods used today. The results show that the acoustic trapping platform is a flexible and gentle cell handling technique and has the potential to become an important tool for cell and particle handling in microfluidic systems. Finally, an all-glass wet-etched device for acoustic continuous flow separations was demonstrated. Previously reported devices have been manufactured in silicon and the possibility to use glass as base material will lower the chip costs, simplifies the fabrication process and decrease the fabrication time.

Key words
Microsystem technology, microfluidics, lab on a chip, particle handling, cell handling, ultrasound, trapping, acoustic particle manipulation, standing waves
To my parents
Cover illustration
A cluster of neural stem cells from a rat, levitated in an acoustic standing wave
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

I  \textit{Trapping of microparticles in the near field of an ultrasonic transducer}
Ultrasonics 2005, 43 (5): 293-303

II  \textit{Dynamic arraying of microbeads for bioassays in microfluidic channels}
Lilliehorn T, Nilsson M, Simu U, Johansson S, Almqvist M, Nilsson J, Laurell T
Sensors and Actuators B - Chemical 2005, 106 (2): 851-858

III  \textit{Characterization of micromachined ultrasonic transducers using light diffraction tomography}
Almqvist M, Torndahl M, Nilsson M, Lilliehorn T
IEEE Transactions on Ultrasonics, Ferroelectrics and Frequency Control 2005, 52 (12): 2298-2302

IV  \textit{Noninvasive acoustic cell trapping in a microfluidic perfusion system for online bioassays}
Analytical Chemistry 2007, 79 (7): 2984-2991

V  \textit{Acoustic Differential Extraction for forensic analysis of sexual assault evidence}
Horsman K M, Evander M, Voorhees J, Nilsson J, Laurell T, Landers J P
Manuscript

VI  Acoustophoresis in wet-etched glass chips
Evander M, Lenshof A, Laurell T, Nilsson J
Analytical Chemistry 2008, Accepted

Papers not included in this thesis:

VII  \textit{Temperature control and resonance mode analysis of a 10MHz acoustic transverse trap for µTAS}
Johansson L, Evander M, Lilliehorn T, Almqvist M, Nilsson J, Laurell T, Johansson S
Manuscript

VIII  \textit{An echolocation visualization and interface system for dolphin research}
Amundin M, Starkhammar J, Evander M, Almqvist M, Lindström K, Persson H W
THE AUTHOR’S CONTRIBUTIONS TO THE PAPERS

I Part of experimental work and evaluation, small part of writing

II Part of planning, large part of experimental work and evaluation, part of writing

III Part of planning, large part of experimental work and evaluation, part of writing

IV Large part of planning, major part of experimental work and evaluation, all writing

V Large part of planning, experimental work, evaluation and writing

VI Major part of planning, large part of experimental work, evaluation and writing
# ABBREVIATIONS

<table>
<thead>
<tr>
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<td>ADE</td>
<td>Acoustic Differential Extraction</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>CFD</td>
<td>Computational Fluid Dynamics</td>
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<td>DE</td>
<td>Differential Extraction</td>
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<td>DEP</td>
<td>Dielectrophoresis</td>
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<td>DRIE</td>
<td>Deep Reactive Ion Etching</td>
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<td>iDEP</td>
<td>Insulator-based Dielectrophoresis</td>
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<td>MIM</td>
<td>Metal Injection Molding</td>
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<td>MST</td>
<td>Microsystem Technology</td>
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<td>nDEP</td>
<td>Negative Dielectrophoresis</td>
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<td>PBS</td>
<td>Phosphate buffered saline solution</td>
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<td>PCB</td>
<td>Printed Circuit Board</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<td>Positive Dielectrophoresis</td>
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<td>PIM</td>
<td>Powder Injection Molding</td>
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<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<td>PMT</td>
<td>Photo Multiplier Tube</td>
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<td>PZT</td>
<td>Lead Zirconate Titanate</td>
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<td>RBC</td>
<td>Red Blood Cells</td>
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<td>STR</td>
<td>Short Tandem Repeat</td>
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<td>μTAS</td>
<td>Micro Total Analysis Systems</td>
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</tbody>
</table>
## CONTENTS

1 INTRODUCTION ................................................................. 1

2 MICROFLUIDICS ............................................................... 3

3 MICROFABRICATION ....................................................... 7

4 PARTICLE AND CELL HANDLING ............................................... 9

4.1 Passive or hydrodynamic trapping .......................................................... 9

4.2 Patch-Clamp ........................................................................... 10

4.3 Dielectrophoretic trapping ............................................................ 11

4.4 Optical tweezers ........................................................................ 14

4.5 Magnetic Trapping .................................................................... 16

4.6 Acoustic trapping ...................................................................... 16

5 ACOUSTIC METHODS ............................................................. 18

5.1 Acoustic theory ........................................................................ 18

5.2 Acoustic impedance .................................................................... 20

5.3 Transducers .............................................................................. 20

5.4 Piezoelectric materials ............................................................... 22

5.5 Matching layers and resonator design ........................................... 24

5.6 Acoustic particle forces ............................................................... 25

5.6.1 Primary radiation forces ........................................................ 26

5.6.2 Lateral radiation forces .......................................................... 28

5.6.3 Secondary forces .................................................................. 29

5.7 Acoustic Streaming ...................................................................... 30

5.8 Particle trapping platforms using acoustic forces ......................... 31

5.8.1 Macro-techniques ................................................................... 32

5.8.2 Micro-techniques ................................................................... 34
6 A NEAR-FIELD LATERAL TRAPPING SYSTEM.......................... 37
6.1 Development of a piezoceramic base plate................................. 37
6.2 Development of acoustic resonator channels............................... 40
6.3 Design and characterization ..................................................... 41
6.4 Dynamic arraying and bioassays............................................... 44
6.5 Fluidic layer materials .............................................................. 46
6.6 Hydrodynamic focusing and particle trapping force..................... 47
6.7 Cell handling and trapped cell viability ..................................... 49
6.8 Acoustic Differential Extraction............................................... 50
6.9 Monitoring of ATP-release from erythrocytes............................. 54
6.10 Alternative designs for microfluidic trapping ......................... 58
   6.10.1 Silicon resonance cavity .................................................. 58
   6.10.2 Rectangular glass capillaries for trapping........................... 61
6.11 Acoustophoresis in wet-etched glass chips ............................. 63
7 CONCLUSIONS............................................................................. 67
8 POPULÄRVETENSKAPLIG SAMMANFATNING .......................... 68
9 ACKNOWLEDGEMENTS............................................................. 69
10 REFERENCES................................................................................. 71
1 INTRODUCTION

A clear trend within the biomedical field today is to seek to miniaturize existing analysis techniques. By downscaling the analytical systems to the micrometer-regime several advantages are foreseen [1]. The volumetric scale-down decreases the amount of sample and reagents used, which can be crucial in some applications. The decreased amounts of reagents will also mean a decrease in cost per analysis. The reduction in length-scale of the systems will also increase reaction speeds due to the shorter diffusion distances [2].

The scale-down will also increase the surface to volume ratio since the volume scales with the cube of the length and the area only scales with the square. This can be taken advantage of by the use of microparticles as the solid phase of the analysis system. Microparticles are usually based on polymers such as latex, polystyrene or melamine and are available in a wide range of sizes down to tens of nanometers. The particles can be surface-modified in many different ways, e.g. by coating the particles with a layer of anti-bodies that makes it possible for the particle to selectively capture proteins that subsequently can be identified by for instance a fluorescent or chemiluminescent assay. The regulation of temperature, often an important parameter in chemical processes, is also improved by the increased surface to volume ratio. Rapid heating or cooling of samples is possible and allows for faster reaction times and can be used in for instance the miniaturized version of the polymerase chain reaction, micro-PCR [3-5].

Due to the small dimensions, any fluids moving through the micrometer-sized channels will exhibit a completely laminar behavior. The laminar flows, treated more extensively in chapter 2, enable an excellent flow control due to the ease of which the flow-paths can be predicted by for instance computational fluid dynamics (CFD). The laminar flows can be used to position reagents, microparticles or cells in precise spatial locations for use in biochemical applications. The predictable flow-paths also make it possible to use fluidic phenomena to perform, for instance, a size-selective sorting as done in the pinched flow fractionation [6-8].

The microfabrication techniques allow for the integration of a vast variety of different sensors, fluidic mixers [9, 10] and valves [5-7], optical elements [11] etc, making it possible to downscale a large laboratory set-up into a single chip [12], a so-called Lab-on-a-chip or micro total analysis system – µTAS [13]. Due to the small dimensions of the miniaturized analysis components, smaller or hand-held instruments are now possible. By using the different functional units available, a serial process can be implemented and thanks to the small footprint of the miniaturized components, a massive parallelization can be performed on chip [14].

The possibility to have high-density arrays with precise fluidic control has been used to perform particle-based bioassays and to study the response of small cell populations or single cells to different stimuli [15, 16]. Fluorescence is typically used in detection of molecules of interest and provides a powerful technique to image what is really happening on a molecular scale [17, 18]. The microsystems can therefore be an attractive alternative to the micro-titers plates and conventional cell culture bottles used today.
However, in order to perform cell and particle assays, methods capable of different kinds of manipulations/unit operations of particles and cells are needed. Cells to be cultured or studied might for instance need to be immobilized or, in the case of non-adherent cells, levitated to avoid contact with channel walls. Other essential unit operations include enrichment, separation and buffer exchange steps. There are several techniques capable of performing cell and particle manipulation by using passive geometries or different externally induced forces. An overview of the different techniques can be found in chapter 4.

This thesis describes the development and use of a technique to immobilize cells and particles in microfluidic systems. The aim of the system development was to perform non-contact levitation of microparticles and cells by the use of highly localized ultrasonic standing waves in a microfluidic channel. The localized standing waves and the microfluidic interface enable a dynamic arraying approach where the solid phase of the system easily can be exchanged by deactivating the standing waves and letting the particles leave the system with a pressure-driven flow.
2 MICROFLUIDICS

Microfluidics deals with the behavior of fluids in channels with micrometer dimensions. The field involves most aspects of working with these systems, such as fabrication as well as theoretical and experimental studies of the chips. Gases and liquids are both fluids and although liquid-based microfluidics is the major part of the field, gases are also used in microfluidic systems [19]. The micrometer-scale channels are mainly characterized by their laminar flows, making it hard to create turbulence or chaotic flows. Laminar flows are in theory completely reversible and a perturbation to a fluidic system, done by for instance a movement of a component, can be made undone if the component retraces its movement in reverse.

A common way of indicating the laminarity of a fluid is by using the Reynolds number [20]. It indicates whether the viscous or the inertial effects dominate a fluid flow and is defined by:

\[ Re = \frac{\rho \cdot u \cdot D_h}{\eta} \]

where \( u \) is the characteristic velocity of the fluid, \( D_h \) is the hydraulic diameter, \( \rho \) the fluid density and \( \eta \) the fluid viscosity. The hydraulic diameter is defined by:

\[ D_h = \frac{4 \cdot A}{P_{\text{wet}}} \]

Where \( A \) is the cross section area of the channel and \( P_{\text{wet}} \) is the wetted perimeter. The hydraulic diameter essentially describes the diameter of a circular tube with equivalent flow characteristics. It is a very useful way of describing flows in non-circular cross-sections and will give an indication on how large part of the cross-section actually is used for an active flow. A circular capillary will for instance have its diameter as the hydraulic diameter. A square capillary will however have its side length as the hydraulic diameter, demonstrating that the flow in the corners of the square will be negligible. So a circular cross-section with diameter \( D \) will behave in the same manner as a square cross-section with a side-length \( D \) as shown in Figure 1.
The cross-over from laminar to turbulent flow is usually considered to be somewhere in the region of values of \( Re \) from 2500 to 1500. In a microchannel, the Reynolds number is typically extremely small. A channel with a cross-section of 100 x 100 \( \mu \text{m} \) and a water based flow of 1 mm/s would result in a Reynolds number as low as 0.1 showing how truly laminar a typical microflow is.

A newtonian fluid, where the viscosity is independent of the flow velocity, will have a parabolic flow profile during laminar flow. The flow at the channel walls will be zero and the maximal velocity will be in the center of the channel \([21]\).

Channels with small dimensions lead to an increased pressure in the channels making it harder and harder to drive a fluid flow the smaller or longer the channel gets. The D’arcy-Weisbach formula \([22]\) describes the pressure change in a circular channel:

\[
\Delta p = \frac{2 f \rho u^2 L}{D_h} 
\]

\( \Delta p \) denotes the length of the channel, \( u \) the average velocity and \( f \) is the D’arcy friction factor, which for laminar flows can be simplified with:

\[
f = \frac{16}{Re}
\]

The laminar flows are extremely useful in microfluidics. The streamline of a flow can easily be predicted and without turbulence, any mixing that occurs is driven mostly by diffusion. As opposed to turbulent/chaotic mixing, diffusive mixing is a very slow process where the time it takes for a molecule with a diffusion coefficient \( D \) to move a distance \( d \) is given by \([20]\):
A typical molecular diffusion coefficient would be around $10^{-9}$ m$^2$/s meaning that a molecule would need approximately 20 seconds to diffuse a distance of 200 µm. In a small microchip, liquids might not spend long enough time in the chip to have time to mix sufficiently and methods to improve the mixing are thus required.

The Peclet-number shows how the diffusion relates to the convective mixing and can be very useful a microfluidic system to estimate the dominating mode of mixing. It is defined as the ratio of the diffusion time and the time for convective transport, $\tau_c$:

$$Pe = \frac{\tau_D}{\tau_C}$$  \hspace{1cm} \text{Equation 2.6}

Thus, in a system with a high Peclet-number, the diffusion is acting slower than the convective transport and the dimensions might need to be further minimized in order to get a diffusion controlled mixing, if so is desired [23].

The slow mixing can be both an advantage and a disadvantage depending on the intended application. Obviously, if the goal is to mix reagents then slow mixing will make things complicated and invoke the need of mixing components to shorten the path-lengths or create turbulence. There are many different techniques, both passive [9, 10, 24] and active [25] (using externally applied forces) to perform mixing in microfluidics systems. Many of these techniques can perform a very rapid and efficient mixing, however a generic mixing technique that is suitable to all applications remains to be found.

If the specific application is aimed at handling particles or cells then the lack of turbulence can be an advantage. If a positioning or focusing of the particles is performed at one part of the chip, due to laminar flows in the fluidic systems, the particles will maintain their positions throughout the system and just follow the streamlines. This will make it easy to transport particles to specific target locations.

A particle with a diameter $D_p$ moving with a velocity $u$ through a fluid with a density $\rho$, has a force acting on it by the surrounding fluid. This force is called the hydrodynamic drag force and can be calculated using the fluid’s viscosity $\mu$ and Stoke’s law:

$$F = 3 \cdot \pi \cdot \mu \cdot D_p \cdot u$$  \hspace{1cm} \text{Equation 2.7}

Stoke’s law is only valid if the particle Reynolds number is below two, the particle is spherical, the particle is not being affected by other particles and the particle has reached its terminal velocity. So in order to do quantitative measurements of the drag force, a very sparse particle concentration or a single particle in a microfluidic flow will satisfy the above criteria. The particle Reynolds number is defined as:
In order to immobilize a particle in a fluid flow, both a lateral and a vertical force component are required by the trapping technique. The trapping force must be able to overcome both the gravity of the particle and the hydrodynamic drag force. Since the gravity scales with cube of the particle radius and the hydrodynamic drag force scales linearly, the gravity will dominate for large particles while the drag force will dominate for smaller particles and high flow rates. The density of the particle will have an important role in deciding when the gravity becomes the dominant force due to the buoyancy of the suspending medium.

As an example, a 1 µm polystyrene particle in a fluid flow of 1 mm/s will experience a gravitational force of 0.3 fN and a hydrodynamic drag force of 0.9 pN. If the particle had a diameter of 100 µm instead, it would experience a gravity of 260 pN and a drag force of 94 pN.

Using Stoke’s law, the particle radius at which the drag force and gravity are equal for a specific set-up can easily be found.

\[
\lim_{\text{limit}} = \sqrt{\frac{9 \cdot \mu \cdot u}{2 \cdot (\rho_p - \rho_b) \cdot g}}
\]

Where \( \rho_p \) is the density for the particle and \( \rho_b \) the density of the suspending medium. For particles with a radius larger than \( r_{\text{limit}} \), the dominant force to overcome will be the gravity and vice versa.
3 MICROFABRICATION

The field of microsystem technology (MST) and microfluidics is dependent on the ability to fabricate small structures with high precision. The fabrication techniques originally used in MST were derived from the electronics industry, which also explains why silicon has been one of the most common materials used. In the recent years, a process development aimed specifically at MST has triggered the use of new materials and opened up new fields of research.

One of the more straightforward ways of processing silicon is that of wet-etching. Wet-etching can be performed either as an isotropic process or anisotropic process depending on the etchant used [26]. When performing an isotropic etch, a mixture of hydrofluoric acid (HF), nitric acid (HNO₃) and acetic acid (CH₃COOH) is commonly used while anisotropic etching usually is done in potassium hydroxide (KOH). In the isotropic case, all crystal planes will etch with the same speed and no care has to be taken to the orientation of the silicon wafer. When performing an anisotropic etching, different crystal planes, denoted for instance <100>, will etch at different rates, making it possible to create structures with perfectly vertical walls and many other geometries, see Figure 2.

![Figure 2](image)

**Figure 2:** With a single mask (A), several different types of etch-fronts can be achieved depending on the etch-process and mask alignment used. In (B) an isotropic etch-front is seen furthest to the left, while the other two etch-fronts are anisotropic and can be etched in <100>-silicon using different mask-alignment.

As opposed to wet-etching there are also dry etching techniques where a reactive plasma is used for etching. If vertical walls with high aspect-ratios are required or care to crystal plane and wafer orientation cannot be taken, deep reactive ion etching, DRIE, is a widely used dry etching process in laboratories world-wide. In industry, the DRIE is not as common but the use of the process is increasing and the parallelity of the process is also increasing with newer instruments presented.
Alternative materials to silicon were sought early on which resulted in the development of a variety of new fabrication techniques. Today, polymer chips are becoming increasingly popular due to ease of fabrication, large scale and high-speed mass fabrication and a much lower cost. There are several methods available, such as micro injection-molding and hot- or cold-embossing that allow for fast and cheap mass-production of polymeric microfluidic components etc.

Glass is another attractive alternative due to its material properties. It is cheaper than silicon (though more expensive than polymers) optically transparent, electrically insulating and chemically inert. Glass is also a very good material for applications using acoustic waves due to its low absorption of sound.

The probably most common way of processing glass within the MST-field is by wet-etching in hydrofluoric acid (HF). This is an isotropic process but depending on the masking material and the composition of the etchant, different tapered walls can be achieved [27-29]. Fabricating parallel batches of glass wafers is possible with wet-etching processes resulting in a high surface smoothness, suitable for e.g. acoustic applications requiring smooth surfaces for reflectors. For more complex structures it is possible to perform DRIE of glass [30]; however, as for silicon processing it is not the easiest way of fabrication and the etch-depths are limited in glass. It is also possible to perform hot-embossing of glass [31] but this requires expensive masters of materials that do not fuse into the glass. Simpler glass structures or via holes can be powder blasted which produces a rather rough surface but may be good enough depending on the specific application [32].

For components that need to withstand more wear and tear than a polymer would be able to, it is possible to produce metal or metal alloy parts using Metal Injection Molding (MIM) or Powder Injection Molding (PIM) [33, 34]. Here, a metal powder is mixed with a polymer binding and injection-molded in the normal way. After the molding, the binding polymer is pyrolyzed, to leave just the cast metal powder object that subsequently undergoes a sintering process. This combination of the classic polymer injection molding with metal-casting and -pressing is a fast and efficient way of producing components with high precision and strength.
PARTICLE AND CELL HANDLING

Cells can be kept alive in vitro for a long period of time and by supplying a correct environment and nutrients, a normal development and culturing of the cells is possible. Cells can in many ways substitute humans and animals in research labs and also help researchers understand how humans react to drugs or other substances on a molecular level. The traditional way of culturing cells in Petri-dishes, cell culture flasks and roller bottles uses the same culture media during long culturing time and has no easy way of removing metabolites or renewing the nutrients in the media unlike the in-vivo situation. Cell-assays are in turn commonly performed in microtiter plates which have an even more stagnant microenvironment for the cells, an especially important issue when performing assays requiring long incubations times [35].

A step towards a more in-vivo-like environment for cell assays is a perfusion-based system. By a continuous exchange of the cell media, fresh nutrients are provided, metabolites are removed and a more stable micro-environment is thus achieved [36-38]. This can be done in microfluidic-based systems where culture wells for both large cell populations and single cells can be integrated in a fluidic system. Adherent cells can be deposited using different techniques in confined areas, grown on the bottom of a channel and monitored using microscopy. As for non-adherent cells, methods to prevent them from making any contact with the channel walls are needed to fully mimic their natural environment.

There are several different techniques used in MST today that enable the immobilization of cells. Some techniques also have the ability to levitate cells and are thus suitable for use with non-adherent cells as well. Many of these methods rely on externally applied forces that in different ways create a force on particles or cells. The following sections contain a short review of the different techniques used within particle and cell trapping.

It should be noted that most of the techniques can not only be used for trapping but also provide means of performing separations in a continuous flow format. Since the main scope of this thesis is aimed at trapping, the different separation techniques are not covered here. Information on how separations can be performed in microsystems can however be found in a recent review on continuous flow separations [39].

4.1 Passive or hydrodynamic trapping

Passive techniques do not require any external forces to work and can be based on filters, fluidic forces or passive trapping structures such as wells etc. A lot of applications can be based on purely passive techniques but they are also somewhat limited in their performance. They can usually not be made selective to different parameters and there may also be a problem with releasing cells or particles from a specific trap location without disturbing the entire chip.

An example of a passive trapping device is the work done by Lee et al. at Berkeley [40]. The authors use a soft-lithography approach to create an 8 x 8 array of cell culture wells
with an addressable chemical gradient generator, see Figure 3. Cells were loaded into the culture wells and were immobilized while being perfused with growth medium. The cells showed a high viability and cell densities higher than the standard techniques were achieved.

![Figure 3: The passive cell culture array from Lee et al. at Berkeley [40]. The image shows a 5 day old culture of HeLa-cells that have been fluorescently stained for either membrane (red) or nucleus (blue). A green fluorescent dextran-solution was used to demonstrate the chemical gradient generator and was infused from left to right in the image.](image)

The loading procedure will however result in a loss of cells and the cell density within each of the culture wells may differ largely. It is also problematic to selectively remove or insert different cell types in the same array.

A vastly different approach by Lutz et al. from Washington University is based on hydrodynamic forces and is named hydrodynamic tweezers [41]. The chip uses external actuation to achieve an oscillating flow and can thus not be considered a truly passive device. The oscillating flow creates a steady re-circulating flow around cylinders positioned in a channel. A cell entering the streaming eddy will experience a weak force that will keep it circulating in a small confined spot around the cylinder.

This technique claim to have lower shear stresses than other trapping techniques and is aimed at single-cell analysis since a very limited amount of cells can be kept in eddies around the cylinders. As with the previous technique it is not possible to selectively trap or release some cells since the trapping mechanism is working on the entire chip at the same time. Also, it is unclear if it is possible to trap and hold smaller particles or cells in the eddies, and only 22 µm particles and slightly larger cells were demonstrated.

Skelley et al. presented a parallel system for cell fusion based on a large array of passive trapping structures [42]. A simple, yet elegant solution that is based on first trapping single cells in a small well and then reversing the flow to move the trapped cell into a larger well. This approach is fast and guarantees that there is only one cell per well. In the next phase, another cell type is perfused through the system and trapped in the wells together with the first cell type. In this step there is risk of getting more than just the wanted two cells per well but compared to the existing techniques of cell fusion the efficiency of above 50 % is a substantial increase. The paired cells can now be fused using either chemicals or electrical fields.

### 4.2 Patch-Clamp

The patch-clamp method is a contact method where the object is held in place by a suction force from a glass pipette. The suction force creates a tight seal around the cell
and to be able to perform electrical measurements, the seal has to have a Giga Ohm resistance. The inside of the glass pipette is filled with different solutions depending on what is to be measured from the cell. An electrode inside the glass pipette can register changes in a voltage or current and give information on the behavior of single ion-channels in the cell membrane [43]. The developers of the technique, Neher and Sakmann, were awarded the Nobel Prize in Medicine in 1991.

The traditional patch-clamp requires expensive equipment and some manual labor. A lot of effort has been put into making the system more parallel and easier to use by employing microfluidics and microtechnology. The most straight-forward way is to planarize the patch-clamp and put it on a microfluidic chip. Li et al. fabricated a PDMS-based planar patch-clamp with surrounding electrodes, fluidics and valving systems [44]. They used a focused jet of nitrogen to define the 2 µm holes in a polydimethylsiloxane (PDMS) film used to hold the cells. The success-rate of actually achieving a Giga-Ohm seal was 24% which is comparable to some commercial systems. Silicon has also widely been used as substrate material for planar patch-clamp. One example is Matthews and Judy at Caltech that combined DRIE holes in silicon with a PDMS fluidic interface [45].

The traditional patch-clamp can also be combined with microfluidics to create a better system. Pihl et al. used a microfluidic gradient generator in combination with a patch-clamp to rapidly scan a cell through different solutions and record the membrane activities with the patch-clamp electrode [46].

### 4.3 Dielectrophoretic trapping

Dielectrophoresis, DEP, was first investigated and named by Pohl in 1951[47]. It is a phenomenon describing the particle movements in non-uniform electric fields and is not to be confused with electrophoresis, i.e. the movement of charged particles in an electrical field. In the first experiments done with DEP, voltages up to 10 000 V were used and the sizes and kinds of particles used were very limited because of joule-heating. The DEP-forces acting on particles are inversely proportional to the cube of the electrode dimensions used, thus making the forces stronger, and the voltage needed, smaller for miniaturized systems.
A dielectric particle will experience a force when inside a non-uniform electric field. The force stems from a polarization of the particle relative to the surrounding fluid. Depending on the dielectric properties of the particle and the surrounding fluid, the experienced force can be directed towards the high-intensity parts of the field, (Positive Dielectrophoresis, pDEP) or towards the low-intensity parts of the field (Negative Dielectrophoresis, nDEP), see Figure 4. Due to these differences in behavior between different particles, dielectrophoresis can be used to separate particles with different polarization [48]. Exactly how the objects will behave depends on how the electrodes are designed and actuated, and a vast variety of different tasks can be performed [49]. The force for a particle with a radius $r$ in a non-uniform electric field with can be expressed as:

$$F_{\text{DEP}} = 2\pi r^3 \text{Re}[K(\omega)] \nabla E_{\text{rms}}^2$$  \hspace{1cm} \text{Equation 4.1}$$

$E_{\text{rms}}$ is the root mean square of the applied electrical field and $K(\omega)$ refers to the Clausius – Mossotti factor that describes the difference in permittivity between the particle and the suspending medium. Since the permittivity of the particles will decide how strong the force will be, particles or cells with different permittivities can be separated.

The difference in polarization is usually frequency-dependent and to be able to perform a separation, the correct frequency that will result in a separation must be found. Also, the conductivity of the medium will affect the DEP-behavior of the particle and the forces can be enhanced by carefully selecting the suspension medium.

To perform trapping with DEP, the most straightforward method is simply to attract particles or cells to the electrode using either pDEP or nDEP [50, 51]. This will however result in forces driving the particles or cells into a surface, something that may
create problems when releasing the objects from the trap later on. A more elegant, but also much more complicated, trapping approach is the use of nDEP-cages [52]. These cages use eight electrodes to define an nDEP field cage that can hold cells or particles contact-free in the surrounding fluid.

Figure 5: DEP trapping of peripheral blood mononuclear cells from a mixture of blood cells [51].

A less complicated trapping approach is the use of insulator-based DEP (iDEP). Here an array of insulator posts is positioned in a channel and the perturbations in the electrical field that they create can be used for streaming DEP or trapping DEP [53]. An advantage with the use of insulator posts is the lack of electrodes that otherwise can suffer from fouling and reduce the performance of the chip. Also, it is possible to use a DC-field for both electrophoresis and the DEP-trapping. The electrical field around the insulator posts provide for both nDEP and pDEP trapping and thus a selective trap is possible and has been demonstrated using live and dead bacteria [54, 55]. Voltages of up to 200 V/mm has to be applied however, and may be a source of sample heating. Also, the lack of addressability lowers the possibilities of performing more advanced cell assays.

An example of an addressable pDEP-trapping chip was presented by Taff et al. [56]. The design used is a circular electrode with the other electrode in the center of the circle. A major advantage of the implementation used in their work is the addressability of individual trapping sites and the slight disadvantage compared to other DEP-trapping techniques is the fact that trapping only can occur at pDEP making it impossible to perform selective trapping based on cell types etc. Individual trapping, sorting and release of 20 µm particles and HL-60 cells were shown in a 4 x 4 array of trapping sites. The trapping strength at an actuation voltage of 2 Vpp was in the range of 240 – 430 pN.
When DEP was first implemented, high voltages were often needed and these could lead to heating of the medium and damage done to cells being manipulated. The miniaturized systems used today do not have the same problems with joule-heating due to the smaller dimensions and thus the lower voltages needed. Care must however still be taken when operating DEP since the frequency used and the electrode shapes may affect cells and cause them to lyse [57]. This can of course be used to switch between trapping and lysing modes and create a system capable of even more cell and particle handling tasks. It is also important to know how a material or cell reacts to different frequencies in order to choose the correct working frequency of the electrical field.

4.4 Optical tweezers

Optical tweezers were developed in the 60s by Askin and uses a tightly focused laser beam to trap and manipulate particles and cells. The technique offers extremely high precision and can be calibrated to measure the force exerted on the manipulated objects [58].

The momentum carried by the photons is transferred to the object and due to the Gaussian profile of the laser beam, the resulting forces are both self-centering and work against gravity, see Figure 7. By moving the laser, the trapped object is moved along and by using multiple lasers complicated patterns can be achieved. The forces achievable with the optical tweezers are usually in the range of a few hundred pN [58].
Figure 7: A schematic image showing the principle of optical tweezers. If a particle is located off-center (A), the light passing through the particle closest to the center will transfer a larger momentum to the particle, resulting in a force directed towards the center of the laser-beam. When the particle is centered in the laser beam (B) the transferred moment will be equal in all directions and the resulting force will be directed upwards, against the gravity.

An initial problem with optical tweezers was the fact that one laser was needed per object to manipulate. This resulted in either a very low number of objects possible to manipulate or extremely complicated set-ups. With the advent of micro-optics it is now possible to use arrays of micro-lenses or grids to create a complicated laser-pattern with a single laser-beam. It is however still a technical challenge to be able to trap a large number of particles that are individually addressable.

An example of the precision of the optical tweezers is the work done by Smith et al. [59]. A plastic microparticle was attached to each end of a DNA-strand. While holding one of the microparticles fixed with an optical trap, the other microparticle was attached to a glass pipette that was slowly exerting a stretching force on the DNA-strand. By monitoring the force measured by the optical tweezers while stretching the DNA it was possible to study the elasticity of the DNA-strand and see at which forces it will lengthen and how much.

Another fascinating display of the achievable accuracy with the optical tweezers is the work of Arai et al. [60]. In order to study the mechanical properties of common filaments in the human body, such as actin, two beads were attached to each end of a strand of material and a knot was tied. By tightening the knot, and thus reducing the radius of curvature, the required rupturing force could be measured and different materials compared.
4.5 Magnetic Trapping

In magnetic trapping, magnetic particles, or cells magnetized by magnetic nanoparticles, are trapped in magnetic fields inside a microchip, see Figure 8. A particle with a magnetic core can be coated with a non-magnetic material that can be tailored to attach to certain binders or antibodies of interest. The magnetic particles used can either be nano- or microparticles depending on the application. An important difference between the particles is that nanoparticles will act as non-magnetic particles as soon as the magnetic field is switched off while microparticles will maintain a certain magnetization and cluster to each other even after the magnetic field is removed [61].

Permanent magnets are more often used than electromagnets due to their higher magnetic strength. A 5 mm permanent magnet would exert a magnetic force of 40 pN on a 1 µm particle whereas a coil would result in a force several magnitudes lower. Electromagnets do however create a more flexible system with the possibility of moving particles easily between different trapping sites.

![Figure 8: A schematic image of magnetic trapping. Magnetized beads or cells are drawn to an external or internal magnet where they will remain until the magnetic field is removed.](image)

Magnetic techniques can be used both for separation and for trapping. An example of magnetic trapping is the work done by Lund-Olesen et al. at MIC [62]. Here, magnetic beads with a diameter of 1 µm were functionalized with DNA and trapped using permalloy magnets in a microfluidic chip. The immobilized particles were perfused with a complementary DNA-sequence and hybridized. By incorporating a staggered Herring-bone mixer in the hybridization channel, a more even number of particles were trapped at each trapping-location and also the diffusion of the DNA into the trapped clusters were improved.

4.6 Acoustic trapping

In acoustic trapping, high frequency ultrasound, typically 1 – 10 MHz, is used to create a standing wave in a microchannel. The pressure differences arising from the standing wave lead to several different forces that can be used to perform selective separations [63] or trapping. The theory behind acoustic forces and its application within trapping is further detailed in chapter 5. The author’s contribution to the field of acoustic trapping is presented in chapter 6.
5 ACOUSTIC METHODS

Ultrasound is used in many different areas from fetal monitoring and measuring to lysing cells and welding. The main difference in the ultrasound used in these vastly different applications is the frequency and the intensity of the sound. In diagnostic ultrasound, frequencies between 2 and 15 MHz are normally used. The lower frequencies are used when increased imaging depth is required and the higher frequencies when high resolution is needed, since higher frequency means smaller wavelength but an increased attenuation [64]. Ultrasonic welding is usually at the other end of the scale, in the 20 - 50 kHz-range and with very high intensities [65]. Ultrasound used for cell lysis is also in the kHz-range and uses high intensities to cause cavitation and break open the cell membranes.

The versatility of ultrasound has given rise to some discussion on whether it is safe to use it to manipulate live cells or not. Can a technique used for welding and cell lysis be completely safe? The answer is yes and there have been numerous studies that show that for higher frequencies and reasonable intensities, no adverse effects on cells can be seen [66, 67]. The frequencies used in medical ultrasound for monitoring fetuses etc are carefully considered and monitored to keep the intensities within safe limits. The ultrasound used in microsystems is usually in the MHz-range and the intensities are kept low and thus do no harm to live cells. Several articles have been published that deal with the effect on cells done by acoustic manipulation in microsystems [68-71]. Most acoustic devices in microsystems utilize standing waves and position cells in pressure nodes where the stress on the cells are minimal and they shouldn’t be affected by the sound field even during long exposures [72].

5.1 Acoustic theory

Sound can be seen as pressure variations travelling in a medium. In our normal everyday hearing, the sound waves are longitudinal waves travelling through air. The human hearing range is usually said to be between 20 and 20 000 Hz and frequencies below 20 Hz are termed infrasound while frequencies above 20 kHz are called ultrasound [73].

A wave can be defined as an equation that solves the so-called wave-equation. For a one-dimensional wave with the displacement $y(x,t)$ and velocity $v$ the wave equation can be written:

$$\frac{\partial^2 y(x,t)}{\partial x^2} = \frac{1}{v^2} \frac{\partial^2 y(x,t)}{\partial t^2}$$

Equation 5.1

Many different kinds of waves can be a solution to the wave equation. A commonly used wave that satisfies the relationship is a harmonic sinusoidal wave, which can be written as:

$$y(x,t) = A \sin(\omega t \pm kx)$$

Equation 5.2
The displacement amplitude, $A$, of the wave, the angular frequency, $\omega$, and the wave-number, $k$, are all constants and the direction of the traveling wave is decided by the sign of the $kx$-term.

Equation 5.2 only describes a traveling wave, whereas most techniques used for particle manipulation instead uses standing waves. A standing wave is formed due to interference between a traveling wave and a reflection of that wave. By superposing the incoming and reflected wave a new wave can be formed where, if the circumstances are correct, the wave is standing still. The standing wave can be written as [74]:

$$y(x,t) = A_{SW} \sin(kx) \cdot \cos(\omega t)$$  \hspace{1cm} Equation 5.3

In the case of a perfect reflection, the maximal amplitude, $A_{SW}$, is twice the amplitude of the incoming wave due to the superpositioning with the reflected wave. The wave is now divided into a stationary part and a time-dependent part, thus creating a fix waveform that varies its amplitude in time. The parts of the wave that have no movement at all are called nodes, as compared to anti-nodes where the wave is oscillating at its largest amplitude, see Figure 9.

\begin{figure}[h]
  \centering
  \includegraphics[width=0.5\textwidth]{figure9.png}
  \caption{A traveling wave pulse superposed with its reflected wave with 1.5$\lambda$ between the reflectors. The distance between two nodes or anti-nodes is always $\lambda/2$.}
\end{figure}

The frequency at which a single node is formed is called the fundamental frequency and occurs when the distance between the reflectors is half of the wavelength. By driving the wave at a specific frequency, a so-called forced oscillation can be created. If this frequency is matched to the fundamental frequency, a build-up in energy will occur, a resonance. By driving a system at or near resonance, large acoustic amplitudes can be built up and the energy required to maintain the vibration is minimal. A resonance will occur for each reflector-distance that is a multiple of a half wavelength. The frequencies at which this occurs are called harmonics and are multiples of the fundamental frequency.

When it comes to particle manipulation and standing waves, it is usually the pressure nodes or velocity nodes that are of interest rather than the displacement nodes. The displacement and pressure is phase-shifted from each other causing the displacement node to be the pressure anti-node and the displacement anti-node to be the pressure node. The velocity nodes occur at the same positions as the displacement nodes and are therefore phase-shifted when compared to the pressure nodes.
5.2 Acoustic impedance

The specific acoustic impedance is a material property defined as the ratio of the acoustic pressure, $p$, and the particle speed, $u$, in the medium [75]:

$$ z = \frac{p}{u} \quad \text{Equation 5.4} $$

Most waves can be approximated as a plane wave as long as the cross-section of the beam is significantly larger than the wavelength. For a plane wave, the specific acoustic impedance can also be expressed using the density of the material, $\rho$, and the sound velocity, $c$:

$$ z = \rho c \quad \text{Equation 5.5} $$

The acoustic impedance is often used to calculate reflection and transmission coefficients in order to determine what materials to use in an acoustic system. The unit for the specific acoustic impedance is $\text{Pa.s.m}^{-1}$ but is often also referred to as Rayl, in honor of Lord Rayleigh. Due to the rather large velocities and densities when expressed in $\text{kg/m}^3$ and m/s, the MRayl is the most common form used.

The reflection coefficient for the acoustic pressure when a wave travels from a material with the acoustic impedance $z_1$ to a material with the acoustic impedance $z_2$ can be defined as a ratio of the acoustic impedances of the materials:

$$ R = \frac{z_2 - z_1}{z_2 + z_1} \quad \text{Equation 5.6} $$

The transmission coefficient can be calculated from the reflection coefficient in turn:

$$ T = 1 - R \quad \text{Equation 5.7} $$

When $R = 0$, a complete transmission will occur and for very large differences in acoustic impedances in the two materials, an almost total reflection will occur. Taking these parameters into account is very important when designing transducers or deciding what materials to use in an acoustic system.

5.3 Transducers

A transducer is used to convert one type of energy, i.e. electrical, into another type, i.e. acoustic. In acoustics, a common way of studying a transducer is by viewing it as a plane, circular piston. This simplification makes it possible to write down analytical solutions for the pressure distribution within certain regions of the sound-beam: the axial pressure distribution and the far-field [75]. If the pressure distribution along the sound-axis is studied, a strong interference effect can be seen in the pressure amplitude. A variation between minima and maxima is seen until a certain distance from the
transducer where the amplitude starts to decrease monotonically. These regions are termed near-field and far-field. The near-field is the region close to the transducer surface that exhibits a complicated acoustic field. The far-field is the region after the last maximum, as seen from the face of the transducer, where a monotonic decrease in amplitude is seen. The near-field positions of the m-th extreme value, starting in the far-field, for a transducer with a radius \( r \) can be described by:

\[
 z_m = \frac{r^2}{m \cdot \lambda} - \frac{m \cdot \lambda}{4}
\]

Equation 5.8

The first extreme value, marking the transition between far-field and near-field can thus be found in position \( z_1 \):

\[
 z_1 = \frac{r^2}{\lambda} - \frac{\lambda}{4} \approx \frac{r^2}{\lambda}
\]

Equation 5.9

A very common situation is that \( \lambda \ll r \) making the simplified expression, seen in Equation 5.9, useable. As can be seen in the equation, a smaller transducer has a shorter near-field. Also, higher frequencies lead to longer near-fields. Figure 10 shows a plot of the first six (counting from the far-field) extreme values seen in the near-field.

![Figure 10: The variations in pressure amplitude in the near-field of a circular transducer with a radius \( r \) [75]. The dashed curve is the far-field approximation valid only outside the near-field.](image)

### 5.4 Piezoelectric materials

An ultrasonic transducer typically uses a piezoelectric material to transform electric signals into pressure variations. In a piezoelectric material, mechanical stress induces a proportional charge, the so-called direct piezoelectric effect. The effect is reversible and
by applying a voltage over a piezoelectric material, a deformation of the material is achieved, the converse piezoelectric effect. These effects are used to create high precision sensors, actuators and high frequency transducers [76]. One of the most common piezoelectric materials used today is the lead zirconate titanate, PZT. It was discovered in 1954 and has since been used in an abundance of different applications. It is also the piezoelectric material used to create the ultrasonic waves in the work presented in this thesis.

By subjecting a piezoelectric crystal to an alternating electrical field, a periodic deforming is created and can be used to produce sound waves of frequencies up to THz. The strain, and thus also the displacement, in a piezoelectric material is usually proportional to the electric field applied [77], making the pressure amplitude of a travelling wave proportional to the voltage applied to the transducer.

To actuate a transducer at its thickness resonance, two thin electrodes usually cover the ends of the piezoelectric material as seen in Figure 11. The typical sound velocity in PZT is around 4000 m/s and to create a strong thickness mode resonance, the thickness usually corresponds to half a wavelength. A piezoelectric crystal with a fundamental resonance of 10 MHz thus has a thickness of 0.2 mm.

![Figure 11: A schematic image of a piezoelectric disc actuated at its thickness resonance (decided by its thickness)](image)

By looking at the force acting on the transducer as a voltage, and the particle movement as a current, an equivalent electrical circuit representing the piezoelectric transducer can be set up as a model, see Figure 12. R, L and C represent the material parameters of the piezoelectric material whereas C₀ is the capacitance of the electrodes. The inductance L is associated with the mass of the piezoelectric material, the capacitance C is associated with its stiffness while the resistance R is associated with the losses during operation of the piezoelectric material [78].
The equivalent electrical network predicts two possible resonances when studying the transducer as a single unit; the series resonance, which corresponds to the thickness resonance, and the parallel resonance, which is the combined electrical resonance of the electrode capacitance and the piezoelectric element.

The resonant behavior of the transducer can be measured with an impedance analyzer. An impedance scan of one of the miniaturized transducers used in the work presented in this thesis can be seen in Figure 13. The series resonance can be seen as the impedance minima at 11.5 MHz and the parallel resonance corresponds to the impedance maxima at 12.2 MHz.
The impedance spectrum shows the uncoupled transducer, as it would resonate without any load. However, when using acoustic forces for particle manipulation, a resonance cavity is used to form a standing wave. How the dimension of the resonance cavity is chosen with respect to the different resonances in the transducer can have a major impact on the behavior of the system, e.g. the coincidence resonance effect [79]. To avoid it, the resonance cavity should not be tuned to the same frequency as the transducer resonance. The effect of coupling these two resonances to each other is a resonance frequency split. The original resonance frequency moves into two separate frequencies, one lower and one higher than the original frequency and the system will now not resonate at the intended frequency.

5.5 Matching layers and resonator design

In order to maximize the acoustic input into a system, the acoustic properties of the materials in the system and of the transducer must be taken into account. A typical piezoelectric element, such as PZT, has a very high density and sound velocity compared to for instance human tissue or water-based buffers used in Microsystems. This mismatch in acoustic impedance will create a very large reflection coefficient, in accordance with Equation 5.6, and thus lead to a very low amount of sound actually leaving the piezoelectric element. To maximize the acoustic output, a so-called matching layer is commonly used in a transducer. This material has an acoustic impedance
somewhere in between the transducer and the target material and can thus reduce the reflection and increase the amount of sound transmitted [64].

An acoustic resonator usually consists of the transducer, a matching layer, the resonator cavity and the reflector, see Figure 14. The dimensions of each of these components will have a major impact on the behavior of the acoustic system. In order to design a resonant system, the desired position of the pressure node must first be decided. Hawkes et al. have made simulations using different thick matching layers, resonator cavities and reflectors showing where the pressure node would be expected to occur in each different combination [80]. If a pressure node in the center of the channel is desired, the most advantageous configuration would be a matching layer with a thickness of a quarter wavelength, a half wavelength resonance cavity and a quarter wavelength reflector. An identical configuration but with no matching layer will also lead to a pressure node in the center of the channel. However, the amount of energy entering the resonance cavity will be much lower without the matching layer.

![Figure 14: The principle design of an acoustic resonator in order to achieve a pressure node in the center of the resonance cavity. The material dimensions are to scale when using stainless steel as matching layer, a water based fluid in the resonance cavity and glass as a reflector.](image)

For the resonators used in this thesis, no matching layer was used, the resonance cavity was a half wavelength and the reflector was an odd multiple of a quarter wavelength. The resonance cavity and the reflector were both designed to match a frequency falling in between the series and parallel resonance of the transducer [78].

### 5.6 Acoustic particle forces

In acoustic particle manipulation, there are several forces acting on a particle situated in the standing wave; the primary radiation force, a lateral force and secondary forces acting between the objects in the standing wave. All of the mentioned forces are important when performing particle trapping in a standing wave. As can be seen in Figure 15, each force plays a vital role in the stages of the trapping procedure. The primary radiation force focuses the particles into a nodal plane and is responsible for maintaining the levitating force, working against gravity. It is also this force that makes the system non-
contact. The lateral forces will center the particles in the sound-field and in the set-up used in this thesis, the lateral forces will also work against the fluidic drag force, thus keeping the particles in the trap in a constant-flow system. The secondary forces will create attractive forces that will keep the particles in a cluster.

Figure 15: A schematic image showing how the different acoustic forces act on a particle suspension in a resonance cavity (a). When the ultrasound is activated and the standing wave is formed, the particles will be moved into the pressure node by the primary radiation force (b). Lateral forces will focus the particles to the center of the sound field and while in the nodal plane, secondary interparticular forces will create an attractive force between the particles (c). The end result is a centered, levitated cluster of particles situated in the center of the resonance cavity (d).

5.6.1 Primary radiation forces
The primary radiation force acts in the direction of the propagating wave and pushes object to either nodes or anti-nodes depending on the material parameters of the object. King [81] was the first to derive an expression for the force acting on a rigid sphere suspended in a plane standing wave. His analysis did not include the compressibility of the particles or the viscosity of the fluid itself. He drew the conclusion that the force acting on a particle from a standing wave was much greater than that of a travelling wave and could thus be used for manipulating objects. Yosioka and Kawasima calculated the force acting on spheres of finite compressibility starting from King’s earlier derivation [82]. They also calculated how bubbles should behave in standing and progressive waves and came to the conclusion that small bubbles should be drawn to the pressure anti-nodes whereas larger bubbles should collect at the pressure nodes. However, for bubbles that begin to resonate in the acoustic standing wave, the radiation pressure should vanish. Gorkov derived the expression again, this time taking the fluid viscosity as well as the compressibility of the spheres into account [83]. His expression for the radiation force on a spherical particle suspended in a standing wave is:

\[
F_{PRF} = 4\pi ER^2(kR)\sin(2kx) \left\{ \frac{\rho_o + \frac{2}{3}(\rho_o - \rho)}{2\rho_o + \rho} - \frac{1}{3} \frac{c^2 \rho}{c_o^2 \rho_o} \right\}
\]

Equation 5.10
$R$ is the particle radius, $k$ is the wave number and $x$ is the particle's position in the wave. $\rho$ and $\rho_0$ is the particle and fluid density and $c$ and $c_0$ is velocity of sound in the particle and the fluid respectively. $E$ is the mean total energy density and can be expressed using the maximum pressure amplitude $P_0$:

$$\overline{E} = \frac{\langle p^2 \rangle}{2 \rho_0 c^2} = \frac{P_0^2}{4 \rho_0 c_0^2} \quad \text{Equation 5.11}$$

The equation can now be rewritten as:

$$F_{PRF} = 4\pi R^2 (kR) \frac{P_0^2}{4 \rho_0 c_0^2} \sin(2kx) \cdot \left\{ \frac{\rho_0 + \frac{2}{3} (\rho - \rho_0)}{2 \rho_0 + \rho} - \frac{1}{3} \frac{c^2 \rho}{c_0^2 \rho_0} \right\} \quad \text{Equation 5.12}$$

The compressibility of a material can be written as:

$$\beta = \frac{1}{\rho c^2} \quad \text{Equation 5.13}$$

By inserting the compressibility into the equation and using the particle volume, $V$, instead of the radius, a more commonly used formulation can be written:

$$F_{PRF} = \frac{\pi P_0^2 V \beta_0}{2 \lambda} \sin(2kx) \cdot \left\{ \frac{5 \rho_0 - 2 \rho}{2 \rho_0 + \rho} \frac{\beta_0}{\beta} \right\} \quad \text{Equation 5.14}$$

where $P_0$ is the peak pressure amplitude from the transducer and $\lambda$ is the wavelength of the standing wave. $V$ is the volume of the particle, $\beta_0$ and $\rho_0$ is the compressibility and density of the medium and $\rho$ and $\beta$ is the density and compressibility of the particle itself.

The primary radiation force is highly dependent on the size of the particle, scaling with the cube of the radius. This makes it possible to use the acoustic forces size-selectively to perform particle sorting based on the particle radius. Equation 5.14 also states that shorter wavelengths result in stronger forces. This makes the acoustic techniques very suitable for miniaturized systems as the force becomes stronger when scaling down the system.

The last term in the equation is called the acoustic contrast factor. The material parameters of the particle and the medium will decide whether the forces will be directed towards the anti-node or the node of the standing wave. By adjusting the density of the medium it is possible to tune a system to create a separation of different objects based on their density or compressibility.
The equation describing the primary radiation force has been derived under the assumption that the particle levitated is relatively small \((kR \ll 1)\) which is not always the case. Using King’s formulation of the primary radiation force, Lierke introduced a size-factor, \(f_1(x)\) where \(x = 2kR\), that scales the radiation force, taking the actual size of the particle into account \([84]\). The size-factor also states that a particle with a size of \(D > 0.715\lambda\) cannot be levitated by the primary radiation force.

\[
F = F_{\text{King}} \cdot f_1(x) = F_{\text{King}} \cdot \frac{3}{x^2} \left( \frac{\sin(x)}{x} - \cos(x) \right) \quad \text{Equation 5.15}
\]

### 5.6.2 Lateral radiation forces

Apart from the primary radiation force, lateral radiation forces are also active. The lateral radiation forces, sometimes called Bernoulli forces, arise from the fact that there is an asymmetry in the wave-front. The derivation of Equation 5.10 is made on the assumption that the wavefront is perfectly planar. If the wave-front really was perfectly planar the particles focused into a node would be free to move around within the node. However, the wave-front from a piezoelectric element is typically divergent with reducing amplitude at the sound beam edges \([84]\). This will result in a lateral force-component that will draw objects into the center of the beam. The relation between the maximal lateral and primary radiation force at the place of maximum primary radiation force \((x = \lambda/8)\) in an open air levitator has been estimated to \([85]\):

\[
\frac{F_{\text{PRE}}}{F_{\text{LAT}}} \approx 2.17 \cdot \frac{2n + 1}{\sqrt{2n + 2}} \quad \text{Equation 5.16}
\]

where \(n\) is the number of the node as counted from the transducer. In the first node the lateral forces would then be approximately 30% of the primary radiation force. To achieve a stable trap, Daidzic recommends that the first and last node in the standing wave should be avoided due to near- and far-field effects \([84]\). Thus, the formulation in Equation 5.16 may not be directly translated to the kind near-field trapping system used in this thesis.

Gröschl estimated that by assuming a constant amplitude gradient and by looking in the vicinity of a dense particle in a pressure node, the lateral force could be written as \([86]\):

\[
F_{\text{LAT}} = \pi \rho \omega^2 R^2 \hat{u}_o \hat{u}_m \quad \text{Equation 5.17}
\]

where \(\rho\) is the fluid density, \(\omega\) the angular frequency and \(R\) the particle radius. \(\hat{u}_o\) denotes the displacement amplitude at the center of the particle and \(\hat{u}_m\) the difference in displacement amplitude at the edge of the particle as compared to the center amplitude.

According to equation 5.17, the lateral forces are not as size-selective as the primary radiation force but instead depend on the square of the radius. Also, the greater the
difference in amplitude between the center of the particle and the edge of it, the greater the force. In order to create a strong lateral trap, a large lateral gradient would be needed.

5.6.3 Secondary forces
The secondary forces, sometimes called Bjerknes or König forces, arise from the scattered field of the main incident sound wave on the objects to be manipulated [86]. The result is a force that can be described by:

$$F_{SEC} = 4\pi R^6 \cdot \left\{ \frac{(\rho_0 - \rho)^2 \cdot (3\cos^2\theta - 1)}{6\rho d^4} \cdot v^2(x) - \omega^2 \rho \frac{(\beta_0 - \beta)^2}{9d^2} \cdot p^2(x) \right\}$$  

Equation 5.18

The center-to-center distance between the particles is denoted $d$ and $\beta$ and $\beta_0$ are the compressibilities of the particles and the suspension fluid. The particle radii is denoted $R$ and $\theta$ is the angle between the center-line of the particles and the propagation direction of the acoustic waves. $V(x)$ and $p(x)$ is the velocity and pressure field at the position of the particles. The equation is based on the assumption that there is no difference in $v(x)$ or $p(x)$ at the different positions of the particles.

The first term of the equation is dependent on the angle $\theta$. If the centerline of the particles is parallel to the wave propagation direction, the first force term will be repulsive. If, however, the particles are perpendicular to the wave propagation, the first term will be attractive, see Figure 16. The second term of the equation is not angle-dependent and will always be attractive.
5.7 Acoustic Streaming

Apart from the acoustic radiation forces, there are also other forces affecting objects in a standing wave. There are several kinds of streaming that will arise in a fluid due to the acoustic wave, so called acoustic streaming. The streaming is caused by the absorption of the acoustic energy by the medium. This energy absorption will manifest itself as a streaming of the medium and arises due to the intensity variations within the acoustic wave [87]. Due to the increased absorption of acoustic energy at higher sound frequencies, the acoustic streaming scales with the square of the frequency and should thus be very pronounced for high frequencies. It also scales with the square of the radius of the sound beam, making it less pronounced for smaller transducers [88].

Acoustic streaming can be divided into Eckart streaming (Quartz wind), Rayleigh streaming or Schlichting streaming (microstreaming) [89, 90]. Eckhart streaming, or Quartz wind, is one of the more common types of acoustic streaming. It usually appears in dimensions much larger than the wavelength and is illustrated by Figure 17a. Rayleigh streaming is induced by standing waves between parallel plane boundaries and usually occurs in dimensions in the wavelength order. The streaming creates vortices with a distance of $\lambda/4$, see Figure 17b [91]. Since the distance between the vortices is the...
same distance as between a node and an anti-node, this type of streaming may impact on the acoustic particle manipulation. The final type of streaming is seen near walls or other objects and creates vortices within a boundary layer much smaller than the sound wavelength. Due to the very restricted range in which this streaming occurs, it is usually of minor importance in particle handling applications.

**Figure 17**: Eckhart streaming (Quartz wind) creating a stream directed from the transducer in (A). Rayleigh streaming with counter-rotating vortices spaced $\lambda/4$ apart in (B).

Apart from the purely acoustically induced streaming, there may also be a convective, thermal streaming in resonator systems. A transducer will generate a certain amount of heat due to mechanical losses in the transducer, and this heat may induce thermal streaming within a fluid.

### 5.8 Particle trapping platforms using acoustic forces

There are many research groups that have been working with acoustic forces during the last thirty years. It is however not until recently that the technique has merged with microtechnology and proven to be a flexible tool for handling cells and particles on chip. The examples listed below all regard trapping/immobilization of particles or cells. A lot of effort has been put into other application areas in microtechnology as well. Acoustic forces have also been used within for instance constant flow separation [70, 92, 93], alignment [94-96] and washing [97, 98].
5.8.1 Macro-techniques

As early as 1874, Kundt used particles to visualize an air-borne standing wave in a tube [74, 99]. A horizontal glass tube was filled with fine particles and a membrane was attached to one of the ends of the tube. By using a speaker, the membrane could be actuated and a standing wave created in the tube. The particles would now assemble at the displacement nodes and thus visualize the standing wave.

However, Kundt’s experiment did not show the potential of levitating or separating particles. Some 30 years ago, NASA and ESA started to develop containerless processing. Several possible techniques existed with acoustic levitation as one of them [85]. These open-air levitators, still in use today, are rather large instruments that create a standing wave in air and make it possible to position and levitate droplets of a liquid or small and light solid matter.

An example of an open-air levitator can be seen in Figure 18. This levitator is designed to work at a frequency of 58 kHz and an optimal drop volume to levitate is 5 µl [100]. Intended research fields for these kinds of levitators are the study of molten materials, liquid drop behavior and microgravity, but they have also found use within chemistry and microtechnology.

![Figure 18: A schematic picture of the DANTEC levitator APOS BA 10.](image)

By using levitated droplets within analytical chemistry, some of the advantages of the microtechnology can be benefited from in a completely containerless system. Droplets between 100 nl and several microliters can be levitated and the chemical reactions confined to these droplets. The levitated droplet chemistry has successfully been used for
single-cell analysis, crystallization studies and sample enrichment [101]. Here, the problem with administering fluids to the sample volume is solved by using piezoelectric dispensers, aimed at the levitated droplet and capable of dispensing pl-droplets with a kHz-frequency [102].

![Figure 19: Levitation of a single droplet in an open-air levitator [101].](image)

The use of radiation forces on particles and cells in liquid-suspensions have been a more common approach than the open-air levitators. Baker showed in 1972 that a band-formation of erythrocytes owing to the radiation forces could be observed when subjecting the cells to a 1 MHz standing wave in polystyrene containers [103]. There has also been considerate interest in using acoustic standing wave devices to aggregate and harvest suspended cells in bioreactors. The idea is to increase the sedimentation rates of the cells and perform a fast cell harvest using the standing waves as an acoustic filter. Coakley et al. examined the band formation and sedimentation rates of erythrocytes at a multitude of frequencies in centimeter long devices [104].

Hertz demonstrated an acoustic particle trap using two opposing focused 11 MHz transducers aimed at a cavity in a water-bath [105]. Stable trapping of 2 µm glass particles and red blood cells were performed and calculations showed that an acoustically trapped particle would exhibit a lower rms-displacement than if trapped with optical tweezers.

Gröschl et al. presented a very nice work with an acoustic filter aimed at fermentation of mammalian cells in bioreactors [72, 106]. The acoustic chamber had a volume of 50 ml and worked at frequencies around 2 MHz. The system was cooled using a low temperature flow close to the resonance chamber and this allowed for stable operating up to power levels of 80 W. There were no signs of effects on the cell viability and the maximum daily harvest was increased by a factor 15 as compared to a system without the acoustic filter. The separation efficiency reported was 99.5 % at a flow rate of 0.5 mm/s, corresponding to a volume throughput of 24 l/day.
Saito et al. used the intersections of two orthogonal standing waves to trap and immobilize locomotive microorganisms, see figure 20 [107]. The chamber used was 10 x 10 x 1 mm$^3$ and the frequency used was around 3 MHz. By changing the frequencies of the transducers slightly, the trapped microorganisms could be shifted from one set of intersections to another and moved to a desired location.

Figure 20: The set-up used by Saito et al. to manipulate live microorganisms. The photos to the right show live Euglena microorganisms that were originally swimming freely. When applying a 3 MHz standing wave in one of the dimensions of the chip, the microorganisms were trapped and immobilized in the pressure nodes. Reused with permission from [107]. Copyright 2002, American Institute of Physics.

In a recent paper, Ruedas-Rama et al. presented ultrasonic trapping in a modified flow cell for IR spectroscopy [108]. The dimensions of the flow cell were 3 x 5 x 18 mm and a frequency of 2 MHz was used to trap clusters of different particles. The Raman spectra from the particles were collected and thus a monitoring of chemical reactions could be performed.

5.8.2 Micro-techniques

Before the acoustic particle manipulation techniques were taken into microsystem technology, capillaries were used to obtain the small trapping volumes wanted. Tilley et al. used microslides with 100 and 300 µm cross-sections although the primary force was acting along the other axis that was 4 cm long [109]. The frequency used was 1 MHz in order to create clusters along the 4 cm long dimension. The set-up was used to study polymer-induced adhesion of erythrocytes, something that would be difficult without immobilizing the cells while still in suspension.

Wiklund et al. used 20 – 75 µm diameter capillaries aligned with the axis of the acoustic wave propagation [110]. A focused 8.5 MHz transducer was used resulting in a 400 µm focused spot size. The system demonstrated the size-selectivity of the acoustic forces by trapping 4.7 µm particles while letting 3 µm particles move along with a constant flow. A version of the system was also integrated with a capillary electrophoresis system to show the potential of enrichment and detection of immunocomplexes [111].
Spengler and Coakley presented a miniaturized system with a 25 µl volume based on a stainless steel spacer configuration in 2000 [112, 113]. The dimension in the sound propagation direction was 250 µm corresponding to a half wavelength at the actuating frequency of 3 MHz. A single fluidic inlet allowed for infusion of a suspension and the device was used to study the aggregation and flocculation of latex particles and yeast cells in water and a salt solution. Bazou et al. later used the same device to further study how the concentration of electrolyte will affect the aggregation of particles in a standing wave [114]. The device was later on redesigned to work at 1.5 MHz and the studies have been aimed at the aggregation and membrane spreading of animal cells in a standing wave, see figure 21 [69, 115, 116]. However, the acoustic trap never used the advantages available in the laminar flow regime and for the studies made a single inlet/outlet was sufficient.

Figure 21: The 1.5 MHz trapping system used by Bazou et al. to study reaction of cells in the acoustic environment can be seen in (A). A cluster of trapped neural cells can be seen in (B). Reprinted from [69] with permission from Elsevier.

Wiklund et al. adapted an acoustic transducer to fit inside a 96-well plate in order to increase the sensitivity in bioassays by concentrating the microparticles [117]. The set-up used a focused transducer with a thickness mode at 4.08 MHz. The transducer was submerged in a well in a 96-well plate, aggregated the particles in several nodes and by turning off the ultrasound all clusters would aggregate at the bottom of the well. By turning on the ultrasound again, all particles in the well could now be trapped in the lowest node, closest to the observation window of the well and imaged using confocal microscopy.
Figure 22: The combined use of acoustic trapping and sedimentation can be used to enrich and collect particles at for instance the bottom of a 96-well plate. Particles are first trapped using a focused transducer in one node and then moved by sedimentation to lower nodes. After several steps an enriched cluster can be immobilized at the bottom node, near the observation window. Reused with permission from [117]. Copyright 2004, American Institute of Physics.

Hultström et al later presented a more microsystem-oriented device. The device used a PDMS-film as a spacer to define the resonance cavity [68]. An external 3 MHz transducer was used to create a standing wave that could perform non-contact levitation of live cells while fresh cell media was perfused through the resonance cavity. Cell cluster of 500 to 2000 cells were trapped and exposed to varying doses of acoustic radiation in order to examine any effect on the viability or proliferation rate of the cells. No direct or delayed adverse effect on the cells could be seen for exposures up to 75 min.

A silicon-glass device for cell and particle trapping working at 2 MHz has also been reported by Svennebring et al. [118]. Here an external transducer is coupled to the chip using an aluminum wedge to create a local standing wave in the channel. The DRIE silicon channel with two inlets and outlets allowed for a perfusion-based system and the external transducer could be positioned anywhere along the main channel. The chip was used to show temperature regulation combined with an acoustic system. The on-chip temperature was monitored using a thermocouple and a temperature regulation with an accuracy of ±0.1°C around 37°C was reported.
6 A NEAR-FIELD LATERAL TRAPPING SYSTEM

The aim of the work in this thesis was to create a trapping platform based on acoustic forces. One of the main ideas was to create a dynamic array for bioassays using particles and cells. The dynamic arraying concept is based on an array of microtransducers in an open fluidic network. The non-contact acoustic trapping is used together with microfluidics to perform parallel bioassays. As an example, antibody-tagged microparticles could be infused, trapped at different locations and perfused with the sample. After a fluorescent read-out of the answer, the ultrasound can be deactivated, the used microparticles flushed out of the system and a new analysis can begin, see Figure 23. By having microparticles as the solid phase, the entire system can be changed by deactivating the ultrasound and the non-contact way of handling will minimize the risk of carry-over.

![Figure 23: The concept of dynamic arraying. Antibody-tagged microparticles are infused (a) and trapped at different locations (b). After sample perfusion, a fluorescent read-out of the answer is performed (c). After the finished assay, the ultrasound is deactivated and the used microparticles flushed out of the system.](image)

To be able to perform trapping of particles and cells in an array-based format in microsystems, several highly localized standing waves have to be set up in the microchannel. The difficulty in creating highly localized standing waves is that ultrasound has a tendency to scatter when passing through materials. If an external transducer is used, the material between the transducer and the actual trapping site has to be very thin and preferably suppress any sound moving in the lateral direction. An alternative design would be to incorporate the transducer into the microfluidic channel itself, which was the method chosen within this project. The piezoelectric transducers used have been operated at around 10 MHz to utilize the stronger particle forces at higher frequencies.

6.1 Development of a piezoceramic base plate

The fabrication procedures and development described here is for the latest version of the trapping platform. The first three papers used a slightly different chip with specialized multi-layer transducers and a different material for the fluidic layer. The main concept, however, remains the same.

The chips used for acoustic particle trapping in this thesis are based on two parts – a glass channel defining the microfluidic network and a platform with transducers, fluidic and electric connections, see Figure 24.
A printed circuit board (PCB) forms the substrate of the transducer platform. By using a circuit milling machine, the electrical connections are isolated with a pointed milling tool, keeping the milled tracks as small as possible. The locations of the through-holes for fluidic access are also marked with the milling machine and finally an air-backing for the transducer is drilled, see Figure 25. The air-backing cavity will function as a total reflecting surface and thus direct all sound produced upwards from the PCB and into the microchannels.

The transducers are based on a 10 MHz Pz26 piezoelectric crystal from Ferroperm Piezoceramics, Kvistgard, Denmark. The piezo is diced into 920 x 920 µm² squares in a silicon dicing machine, see Figure 26, and glued to the PCB, covering the air-backings. Three transducers are used to have the possibility of using three trapping sites, but also to have some redundancy in the system. A conductive epoxy is used and by diluting it with methanol, a lower viscosity and thinner epoxy layer can be achieved. Care is taken when positioning the transducers on the PCB to make sure that there is an equal amount of epoxy under each transducer, as they otherwise will differ in resonance frequency. To avoid short-circuiting of the transducer, no conductive epoxy is allowed to touch the sides of the transducer. Square pieces from a 250 µm thick metal sheet are also glued to the corners (marked with a circle on the PCB) to define the thickness of the epoxy-layer in the next process step.
The PCB with the transducers is covered with a clear epoxy (EPO-TEK 301-2) to create a flat top surface. The chip is placed with the transducers facing down on a piece of flat glass covered with a PTFE-film. The back-side of the chip is covered with another piece of PTFE-film and glass, and a weight is put on top of the entire construction. The epoxy is now cured in an oven at 90°C overnight and the PTFE-film is peeled from the chip. If there are any bubbles around the transducers, they are filled with epoxy and everything is cured for at least another 12 h. When the epoxy is cured, the fluidic access holes are drilled with a 0.7 mm drill and the corner-holes for the brass holder are drilled with a 2.1 mm drill.

The chip is wet-polished using sandpapers against a flat surface. When the silver electrode of the transducers is no longer covered with epoxy, a 2500 grit sandpaper is used to just barely remove the silver and any deeper scratches from the polishing. The polished chip with fluidic holes can be seen in Figure 27.

A new top electrode is evaporated on the chip using an E-beam evaporator. A 40 nm thick layer of titanium is first deposited, followed by 150-200 nm of silver. The chip is masked with tape to ensure that no silver is deposited in the fluidic holes as that might cause a short-circuit. Finally, electrical wiring is soldered to the PCB and glued with conductive epoxy to the silver top electrode, see Figure 28.
6.2 Development of acoustic resonator channels

The wet-etching process used for the glass channels in this thesis starts off with a 4 inch borosilicate glass photomask blank with chromium and positive photoresist. The mask is exposed by a laser lithography system, developed and the chromium is removed with chromium etchant to expose the glass where it will be etched. The photomask is hardbaked for an hour at 120°C before being submerged in an HF:HNO$_3$:H$_2$O mixture (100:28:72) on a rocking table. The glass will etch at roughly 1.3 µm/min in room temperature with proper mixing and fresh etchant. Channels down to a depth of 200 µm can be etched without problems in room temperature. After that, the HF will start attacking the photoresist and create pits and cracks where the glass will start etching as well. If deeper etching is needed, a slight increase in temperature will increase the etch-rate and it should be possible to etch deeper before the photoresist is attacked. It is also possible to use white crown glass instead of borosilicate glass as it has an etch-rate of roughly 4 times that of borofloat making it possible to etch all the way through a 700 µm wafer without causing any degradation in the masking material. White crown will however not bond to silicon or borosilicate glass, so if an all-glass chip is intended, both parts of the chip must be made from white crown.

The etched wafer can now be diced and drilled if needed. Afterwards the photoresist is removed with acetone and ethanol. If the glass will be bonded, the chromium is removed as well; otherwise it is easier to leave it on as it will help in aligning the channels to the fluidic holes on the PCB, see Figure 29.
6.3 Design and characterization

To perform a flexible two-dimensional trapping in a microfluidic environment, an array of miniature transducers would be needed. Lilliehorn et al. developed a fabrication method for multi-layer PZT-elements with dimensions down to 350 x 350 µm² aimed at being used for particle manipulation [119].

A version of these miniature PZT-transducers was used in paper I, to demonstrate the possibilities of performing particle manipulation with a transducer integrated in the fluidic channel [120]. The transducers used measured 800 x 830 µm² and had a fundamental thickness resonance at 10.6 MHz. Microfluidic networks were designed and fabricated in SU-8 with different dimensions to match different transducer resonances in order to find a stable trapping configuration.

The transducers were mounted on a PCB, in a similar manner to the fabrication procedure described earlier, and cast in epoxy. A schematic side-view of the different layers of the device can be seen in Figure 30. The fluidic layer was placed on-top of the PCB and clamped in place with a brass holder to create a sealed channel, see Figure 31.

Figure 30: A schematic side-view of the device showing the transducer, positioned over the air-backing. The transducer is cast in epoxy and a microfluidic channel structure is placed on top of the chip in order to attain a sealed channel. When a suspension of cells or particles enters the standing wave over the transducer, they will be retained until the ultrasound is deactivated.
Figure 31: An early generation of the near-field trapping device. The PCB housing the transducers and electrical and fluidic connections is seen in (A). On the back-side of the chip, silicone tubing is glued to the drilled holes to provide access for standard 1/16” Teflon tubing. The assembled chip with holder and fluidic channel can be seen in (B) with the trapping area showing an array of three transducers visible in the center. On this chip, SU8-channels were used as opposed to the glass-channels described earlier.

A stable trapping of polyamide particles could be achieved when matching the channel height to $\lambda/2$ of the frequency used. If the channel resonance was matched to the transducer resonance, a frequency split was observed as expected. The chip was therefore actuated slightly above and below the resonance frequencies at 9.6 MHz and 11.7 MHz, where stable trapping was observed, see Figure 32.
Figure 32: Trapping of 5 μm polyamide particles. In (a) a particle suspension fills the channel and in (b) the ultrasound has been activated. When applying a flow from right to left, particles that are not trapped are transported away with the flow (c). Upon deactivating the ultrasound, the trapped cluster follows the flow (d).

As can be noticed in Figure 32, a periodic pattern can be seen in the trapped particles. The close proximity of the transducer surface to the objects manipulated will result in a pronounced near-field pattern and several local minimum and maximum over the transducer. This might be negative when an exact location of the trapped object is needed and there are several possible pressure minima in which an object might be trapped. On the other hand, the multiple pressure minima over a small transducer should result in a larger pressure gradient and thus a stronger lateral trapping force.

To verify that indeed the near-field pattern caused the patterned trapping, an optical diffraction tomography measurement, described in more detail in paper III, was compared both to a simulation of the acoustic field and to the actual trapping pattern, see Figure 33 [120, 121].
The near-field leads to several local pressure minima and the transducer area thus hold several local trapping sites instead of just one. By dividing the already small transducer area into even smaller trapping areas, a larger lateral pressure gradient should arise. Since the lateral pressure gradient is responsible for keeping the trapped objects immobilized in a fluid flow, the near-field pattern should lead to a trap withstanding higher fluidic drag forces.

### 6.4 Dynamic arraying and bioassays

In paper II, a device identical to the one used in paper I was utilized to further characterize the trapping and approach the dynamic arraying concept [122]. Dynamic arraying was aimed at performing bioassays in a non-contact mode using an array of transducer. To prove the feasibility of such a system, a bioassay using a single trapping position was performed and the influence of the fluid velocity on the trapped cluster size was investigated.

The original dynamic arraying concept was to use an open fluidic network to address the trapping array. When an assay using several steps is wanted, an alternative approach is to move particles between different trapping positions instead. To show the possibility of moving particles between different trapping sites, an array of three transducers was used. Fluorescent particles were trapped at the first transducer while keeping a constant flow moving from top to bottom in Figure 34. By activating the center transducer when the first transducer was deactivated, the particles moved with the flow and were trapped at the center transducer. By repeating the same procedure, the particles can be moved around between different trapping sites. Channels orthogonal to the main flow channel are present at each trapping site. Using these channels, each trapping site can be addressed with a different reagent or stimuli and a multi-step analysis can be performed.
Using biotinylated microparticles and FITC-labeled avidin, a model bioassay with a fluorescent read-out was performed. The microparticles were trapped over a single transducer and were slowly perfused with the avidin from one of the orthogonal channels, see Figure 35. Fluorescent images were taken during the perfusion and the average intensity of the trapped particle cluster was calculated and plotted against the perfusion time. The increasing intensity shows the binding process of the avidin to the biotin-particles. The avidin-flow was substituted for a washing flow after 100 s to remove any unbound avidin from the trapping region and the remaining fluorescent response $\Delta$, indicated in the graph, shows the amount of avidin bound to the particles in the trap.
Figure 35: Biotinylated microparticles were supplied from the main channel and trapped over one of the transducers. By infusing FITC-labelled avidin from an orthogonal channel a bioassay was performed. The increase in fluorescent intensity shows the binding progress during the perfusion. The avidin was switched to a washing flow to remove any unbound avidin from the trapping region, leaving the fluorescent response $\Delta$.

6.5 Fluidic layer materials

For the work in paper IV, some changes had to be made to the fluidic design and the materials used for defining the fluidic layer. A particle manipulation platform needs to be able to work with both particles of different sizes and living cells. Depending on the immobilization technique used, cells may be affected and it is vital to be able to show that the method used is not harmful to living matter. When working on cell handling, longer handling times than used in a particle-based bioassay are often needed. If targeting a cell with a drug candidate for instance, it takes some time for the cell to metabolize the drug and for any possible side effects to take place.

While working with longer exposure times, a noticeable decrease in trapping performance was seen after some hours. The degradation in performance was due to swelling of the SU-8 when in contact with water and ultrasound for longer periods. Until then, there had been no problems with the SU-8 channels. However, the fabrication process was difficult and the repeatability in thicknesses was not as high as required.

Polyimide was tested as an alternative material. The material was bought in rolls of films with a predefined thickness and could be laser-cut and laminated to a glass reflector. The material had potential but also showed problems with maintaining the predefined thickness during longer trapping periods.

Since polymers showed problems either with swelling or had a high auto-fluorescence, a different type of material needed to be tested. Silicon is a very well-documented material as it is the basis of both microelectronics and microfluidics. By etching channel structures in silicon and performing an anodic bonding to the glass reflector, a stable fluidic layer was achieved, see Figure 36. The accuracy in the fabrication process was
high and no problems with longer exposures to ultrasound were noticed. However, the fabrication process was very time-consuming with many different steps.

![Image](image1.png)

**Figure 36:** An anisotropically etched fluidic layer in silicon, anodically bonded to a borosilicate glass reflector.

By wet-etching the fluidic channels directly in the reflector layer, the fabrication time could be significantly reduced. The wet-etching in glass has the same high accuracy as the silicon etching, but with fewer fabrication steps and no need for bonding, see Figure 37.

![Image](image2.png)

**Figure 37:** A wet-etched glass channel with the masking chromium layer still intact.

During the process of evaluating new materials for the channel/reflector structures, several changes in the microfluidic design were implemented which also explains the difference in channel-layout in Figure 36 and Figure 37. One of the major improvements was the implementation of hydrodynamic focusing.

### 6.6 Hydrodynamic focusing and particle trapping force

When performing trapping on a sample with a low number of cells or particles, it is vital that as many as possible of the objects are trapped. An issue with the earlier versions was that the sample containing the particles covered the entire fluidic channel and thus had the possibility of moving past the transducer along its edges and sometimes even into the
orthogonal channels. By using the concept of hydrodynamic focusing [123] all incoming sample can be directed towards the center of the transducer to ensure a high trapping efficiency, see Figure 38.

**Figure 38:** An injection of blue dye as seen in a trapping zone with its orthogonal channels (A). The blue dye is slowly moving into the orthogonal channels and by using hydrodynamic focusing (B), the inlet can be focused to pass over the center of the transducer, seen as a square in the silver background. This will ensure that all infused material is trapped and the risk of carry-over is reduced.

A comparison between the number of particles lost with and without hydrodynamic focusing was made in paper IV [71]. Roughly 2000 particles were infused into the trapping region by using a flow-rate of either 1 µl/min or 3 µl/min. The amount of particles that were not trapped was manually counted. The result, seen in Table 1, show a high trapping efficiency even without the focusing but a clear advantage with the focusing system can be seen for the higher flow rate.

**Table 1:** The number of particles not trapped when infusing roughly 2000 particles with and without hydrodynamic focusing.

<table>
<thead>
<tr>
<th></th>
<th>Number of particles lost without focusing</th>
<th>Number of particles lost with focusing</th>
</tr>
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<tbody>
<tr>
<td>Low flow rate</td>
<td>26.8 ± 13.3</td>
<td>1.33 ± 1.97</td>
</tr>
<tr>
<td>High flow rate</td>
<td>62.8 ± 13.1</td>
<td>0.67 ± 0.82</td>
</tr>
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An estimate of the maximum trapping force at a voltage of 7 Vpp over the transducer was made using single particle trapping. A single 10 µm polystyrene particle was trapped at a low flow-rate and the flow-rate was sequentially increased until the particle was lost due to the increased fluidic drag force. By calculating the fluidic drag force a measure of the trapping force before that particle was lost can be achieved. A similar approach has been used by Kuznetsova [124] and Tuziuti [125] and shown good agreement with theory.

The trapping force was calculated to be 430 ± 135 pN, corresponding to a linear flow rate of 4.6 ± 1.4 mm/s. The near-field effects might explain the rather large spread in the
forces calculated. It is possible that the particles were situated in slightly different local pressure minima and thus experiencing a slightly different fluidic drag force as well.

6.7 Cell handling and viability of trapped cells

In paper IV, the aim was to investigate any effects that the levitation in a standing wave might have on a living cell. Yeast cells were cultured while suspended in a standing wave in the previously described device. The yeast cells were continuously perfused with cell medium to facilitate cell division and growth. During a time-span of six hours, a clear increase in the amount of cells can be seen in Figure 39. This indicates that the cells were viable and able to divide into new cells, still expressing the yellow fluorescent protein they have been genetically modified to do. This result goes in contrast to the result of Radel et al. [126], where rearrangement of internal components in yeast cells after sonication was observed. The reason for this discrepancy is not clear; however the system used in their experiments is a large macro-system with a much lower frequency and a significantly higher power-input.

![Figure 39: A trapped cluster of live yeast cells is continuously perfused with cell medium to promote growth. During the course of six hours, a clear increase in the amount of cells in the cluster can be seen. This indicates that the ultrasonic environment does not affect the proliferation capabilities of the cells.](image)

A viability assay was also performed on neural stem cells from rats, HiB5-GFP, genetically modified to express a green fluorescent protein. The cells were levitated in the ultrasonic trap and perfused with a phosphate-buffered saline solution for fifteen minutes. The cell cluster was then perfused with acridine orange through one of the side-channels. Acridine-orange uses an active transport into the cells and attaches to living RNA and DNA. The fluorescence from acridine-orange will only be visible in live cells.

In Figure 40, the original cell fluorescence can be seen in (a) while (b) depicts the cell cluster after the acridine-orange infusion. The exposure time in (a) is six times longer
than in (b), illustrating the dramatic increase in fluorescence, thus showing that the cells are viable.

Figure 40: A levitated cluster of neural rat stem cells, HiB5-GFP before (a) and after (b) a viability assay. The exposure time in (a) is six times longer than in (b) and the intensity increase shows that the cells are viable.

6.8 Acoustic Differential Extraction

Within the forensics field, the evidence from rape cases is particularly problematic due to the time-consuming and labor-intensive sample preparation method used. When analyzing the evidence from a sexual assault, a sample is taken from the victim in the form of a vaginal swab, containing both female epithelial cells from the victim and sperm cells from the perpetrator. The epithelial cells are very fragile and will break both when the swab is taken and when the cells are eluted from the swab. This means that the sample will contain a mixture of intact epithelial cells, sperm cells and free female DNA. To attain a complete DNA-profile of the perpetrator, the DNA from the female must be separated from the male DNA, a so called differential extraction (DE) [127].

In the sample preparation method used today, a mild lysis that lyses all the epithelial cells but leaves most of the sperm cells intact is performed. This is done with the buffer used in conventional DE as developed by Gill et al. [128]. The sample is then pipetted into microcentrifuge tubes and centrifuged. The pelleted sperm cells are removed, resuspended in clean buffer and re-centrifuged. This is done a number of times and eventually an enriched and clean male fraction is achieved. The purity of the male fraction is usually somewhere between 70-80 % male. Differential extraction is very labor-intensive and requires many manual steps. The actual processing time for a single sample can be many hours and the technique is not easily automated or integrated with the subsequent analysis steps. By moving the differential extraction to a microchip, the sample preparation should be able to be performed faster, automatically and the DNA from the microchip can be directly integrated with further analysis steps.

In paper V, a new extraction method using ultrasonic trapping is proposed. The method, termed Acoustic Differential Extraction (ADE), is based on a similar chip as
used in earlier papers. The fluidic system has been re-designed to incorporate new features and the multi-layer transducers have been replaced with diced commercial PZ26-elements from Ferroperm, Denmark. The main advantage in using single-layer diced 10 MHz transducers as opposed to the specialized multi-layer transducers from Uppsala University is the time saved in the fabrication of the chips. However, the assembly is slightly more complicated than before. The dimensions of the transducers have also been increased to 920 x 920 µm² to create a larger trapping volume.

The fluidic system was re-designed to incorporate laminar flow valving [129], a simple way of using the control of laminar flows to avoid external valving. The fluidic layout can be seen in Figure 41, and by controlling the ratio of the inlets G1 and G2, the sample flow can be directed either to the male or the female outlet.

![Figure 41: The fluidic design of the ADE-chip. The sample is infused through inlet (S) and hydrodynamically focused by the buffer inlet (B). The flow ratio of inlets G1 and G2 will decide if the sample will go to the male or female outlet.](image)

In the first phase of the extraction, the sample is infused through inlet (S) and directed towards the female outlet. The ultrasound is activated and any sperm cells passing the transducer will be retained and enriched. After six minutes of sample infusion the trapped sperms are washed with pure buffer for two minutes. The flow is still directed to the female outlet where the free female DNA will be collected. After the washing, the ratio of the G1 and G2 inlet is reversed and the main flow is directed towards the male outlet. After a flow stabilization of thirty seconds the ultrasound is turned off. The sperm cluster is released to the male outlet during five minutes to make sure that all sperm cells exit the system regardless of any dispersion.

The total analysis time for the ADE is thus thirteen minutes to compare to the several hours needed for a conventional DE. The ADE-set up is also possible to automate and to integrate with further analysis steps downstream.
The laminar flow valving was modeled and simulated in COMSOL Multiphysics in order to optimize the flow characteristics before implementing the design, see Figure 42. The two-dimensional model used a transient Navier-Stokes module to describe the microfluidics and the Convection-Diffusion module to simulate the female cell lysate. A post-processing particle tracing was used to simulate the movement of the sperm cells upon release from the ultrasonic trap.

A concentration was infused through the sample inlet (S) and focused using the buffer inlet (B), thus simulating the female cell lysate entering the chip. The ratio of the buffer inlets G1 and G2 was modulated to steer the sample to either the female or male outlet. Different channel designs and flow ratios were tested before choosing the design later implemented.

![Figure 42](image)

**Figure 42**: The complete simulated geometry showing the inlets and outlet is seen in (a). The inserts show the velocity flow lines for the flow controlling buffer inlets G1 and G2. In (b) the particles are fixed, simulating the trapping phase, while the cell lysate is injected and directed to the female outlet. Insert (c) shows the velocity field after switching the flow ratio of G1 and G2, now directed the released particles to the male outlet.

To further test the system, a separation of blue dye and microparticles was performed, see Figure 43. A mixture of Evan’s blue and blue polystyrene particles was infused for roughly one minute. The microparticles represented the sperm cells and the blue dye represented the female DNA. The particles were trapped at the transducer while the blue dye went to the female outlet. After the sample infusion was turned off, the blue dye was
washed away, making the particles more visible. The laminar flow valving was switched, directing the fluid to the male outlet and the particles were released. The system appeared to be stable and the laminar flow valving worked as expected.

![Figure 43](image)

**Figure 43:** An image series of a simulated sexual assault sample using Evan’s Blue to represent the female cell lysate and 10 µm polystyrene microparticles to represent sperm cells. In (a), a mixed sample of Evan’s Blue and microparticles is infused into the device. The beads are trapped above the transducer, and can be seen as a dark cluster over the squared transducer area, while the coloring agent is washed into the right outlet. After 1 min of sample infusion, the sample flow is switched off and the channels are washed with buffer (b). When the washing is completed, the outlet flow is redirected to the left outlet. The movement of the dark cluster between (b) and (c) indicates the release of the particles from the acoustic trap and in (d) the microparticles follow the buffer flow to the left outlet.

The performance of the ADE-technique was tested using a biological simulated sample consisted of buccal epithelial cells and sperm cells. 12 µl of semen was diluted in water and added to 500 µl buccal cell lysate in Gill Buffer. The sample was infused for six minutes and washed for two minutes before being released to the male outlet.

The amelogenin gene appears on both the x- and the y-chromosome and provides a way to perform gender identification in DNA-samples. When looking at this specific peak, a female (with two x-chromosomes) is expected to show a single peak. A man (with one x- and one y-chromosome) will on the other hand have two amelogenin peaks. In a mixture of male and female DNA, the ratio between these two peaks will give information on the amount of male to female DNA in the sample.

The DNA in both the female and male outlet was extracted using a QIAGEN-kit and analyzed off-line using an ABI PRISM® 310 Genetic Analyzer. The original sample had a male DNA fraction of 26 ± 2.0 %, Figure 44, and the final enriched and washed male fraction had a purity of 92 ± 7.9 %, Figure 45. The female fraction after ADE had a male component of 5 ± 4 %. Thus an almost fourfold enrichment of the male fraction was obtained in only thirteen minutes. The amelogenin peaks in the STR-profiles have been circled in the figures.
Figure 44: The STR-profile of the original sample showing a male component of 26 ± 2.0% with the amelogenin peak circled. The peak shows a very large preference in one of the two-locations, thus indicating that the sample is mainly female.

Figure 45: The male outlet after ADE, showing a male component of 92 ± 7.9% with the amelogenin peak circled. The two amelogenin peaks are now almost equal in height, indicating a primarily male sample.

6.9 Monitoring of ATP-release from erythrocytes

Red blood cells are known to contain large amounts of adenosine triphosphate, ATP. It has recently become clear that the ATP in the erythrocytes is part of a system controlling the vascular diameter of the blood vessels [130]. The ATP triggers a release of nitric oxide (NO) from the endothelial cells in the blood vessel, and the NO will in turn cause the smooth muscle cells in the vessel to relax and thus widen the vessel diameter, see Figure 46.
The ATP is believed to be released from the cells during physical stress, elevated temperatures or low oxygen levels. There are probably even more factors that can trigger the release from the cells but the study of this release is complicated by the fact that the cells are very sensitive to physical stress. By adding any kind of physical probe or liquid into the cell suspension, a direct response to the stirring or movement of the liquid will be recorded and it will be hard to isolate the reaction from the stimuli itself.

![Diagram of ATP release](image)

**Figure 46:** When subjected to mechanical stress or other stimuli, the erythrocytes will release ATP. The ATP will trigger a release of NO from the endothelial cells that in turn will relax the muscle cells and thus increase the blood vessel diameter.

A microfluidic approach to the study of the dynamics of ATP-release from the erythrocytes should give a more stable environment and make it possible to isolate different factors that may affect the release of ATP. A continuous flow approach has been used by Price et al. to study the effect of a decreasing vessel diameter in a microchannel [131, 132]. The detection method used in their approach is based on the chemiluminescent reaction between luciferin and ATP, see Figure 47, where the interaction between the two molecules results in a photon flux. The light produced can then be monitored using a sensitive photomultiplying tube (PMT).

![Chemiluminescent reaction](image)

**Figure 47:** The detection system used for monitoring the ATP-release. By infusing a luciferin-luciferase-mixture on chip, a chemiluminescent reaction will create a light intensity proportional to the amount of ATP. By monitoring the light intensity with a PMT connected to a computer, long-term continuous monitoring of the ATP-release is possible.

By using the acoustic trapping platform, a small cluster of live red blood cells can be levitated and probed with different chemical stimuli [133-135]. The laminar flows allow
for an efficient way of administering drugs or changing the micro-environment of the cells and by using chemiluminescence, a sensitive continuous monitoring of a small number of cells is possible. The non-contact method of trapping should provide a gentle way of immobilizing the cells with a low base-level of mechanical stress where any additional stimuli should be discernable.

A photography of the rotating holder with a microscope and a PMT can be seen in Figure 48. The trapping chip is mounted on the back-side of the holder to shield it from light. By rotating the holder, either the PMT or the microscope can be used to observe the trapped cells. The PMT output is a digital pulse for each photon it detects and by using an Agilent Counter controlled by a LabVIEW-program the number of pulses for different time-intervals can easily be monitored. A HP waveform generator actuates the chip and two syringe pumps control the fluidics.

![Figure 48](image)

*Figure 48: A picture of the experimental set-up used for monitoring the ATP-release from erythrocytes. A PMT (right) is used to measure the amount of light produced by the luciferin/ATP-reaction and the pulses sent from the PMT are recorded using a counter and a computer. A USB-microscope (left) allows for optical access and monitoring of the erythrocyte cluster. The chip is attached on the back-side of the holder and by rotating the holder, either the microscope or the PMT can monitor the trapping site.*

To verify the detection principle, luciferase in the form of firefly tail extract and D-luciferin was mixed in MQ-water. Red blood cells suspended in PBS were introduced to the system and trapped. Excess RBCs were flushed out of the system using PBS and the trapped cluster was perfused with the luciferin-luciferase-mixture.
Due to the big difference in osmotic pressure between the cells and the MQ-water, the cells were lysed and emitted a large amount of ATP. The signal detected during this experiment can be seen in Figure 49 and seemed to verify the system set-up.

![Figure 49: Measurements on a trapped cluster of erythrocytes that is perfused with a MilliQ-based luciferin-mixture. The high photon counts indicate that the cells are lysed due to osmosis and a large amount of ATP is released as the cell membranes break.](image)

The luciferin and luciferase was now mixed with PBS to provide a physiologically correct buffer for the cells. The result can be seen in Figure 50 and shows that a base-level of 400-500 counts/s is established. Compared to the maximal intensity shown in the previous figure, this is a very low intensity and the detection limits seem promising for the intended studies.

![Figure 50: A measurement on a trapped cluster of live erythrocytes being perfused with a PBS-based luciferin-mixture. A base level of about 400-500 counts/s is established after a couple of minutes.](image)
6.10 Alternative designs for microfluidic trapping

The concept with a trapping platform consisting of a separate fluidic and transducer layer has been proven successful. However, in order to utilize the trapping concept within fields such as forensics, a different design is probably needed. When dealing with DNA-analysis, it has to be absolutely certain that no DNA from any other source than the sample can come in contact with the chip, making a sealed chip a more viable alternative. Also, the process of applying the fluidics layer on the PCB cannot be reproduced exactly, creating a slight variation in resonance frequencies and the local distribution of the trapped clusters. Multi-use chips are also not an option when considering industrial applications and thus a sealed, preferably single-use, design will be needed.

Two alternative designs have been investigated and tested: a silicon cavity etched with Deep Reactive Ion Etching (DRIE) and a rectangular glass capillary with a surrounding fluidic system in Poly(methyl methacrylate) (PMMA).

6.10.1 Silicon resonance cavity

The silicon resonance cavity, seen in Figure 51, is actuated by an external transducer. To create a localized standing wave, a cavity is etched with lateral dimensions matching a half wavelength of the actuation frequency. The structure is etched with DRIE and a borosilicate glass is anodically bonded to seal the chip. A transducer is positioned on the back-side of the chip and creates a lateral standing wave in the cavity. The cavities were 200 µm deep and had lateral dimensions of 375 µm to match $\lambda/2$ at 2 MHz. The inlet and outlet channel of the cavity was made small, 50 µm, to minimize the disturbance of the resonance.
Three different cavities were tested, a round, a square and a hexagon, see Figure 52 [134, 136]. The square cavity had a resonance frequency at 2 MHz as expected. It is also the simplest of the three structures and is most easily coupled to the theory. The hexagon has a resonance at 2.14 MHz although the resonance is not as clear as for the square cavity. The circular cavity had an even higher resonance frequency of 2.35 MHz.
The trapping strength in the different cavities was tested using a trapped cluster of 5 µm polyamide particles. The flow-rate at which the cluster could no longer be retained in the cavity was observed and used as a measure of the trapping strength. The circular resonance cavity showed a much stronger trapping force than the other cavities. At a voltage of 15 Vpp over the transducer, the circular cavity could trap particles against a flow of 2.2 µl/min compared to 1 µl/min for the hexagon and 0.5 µl/min for the square cavity. The latter two cavities showed more acoustic streaming than the circular and that might be part of the explanation to why the circular cavity showed a stronger trapping force.

A force calibration was performed on the circular cavity to see how it compares to the force calibration performed in paper IV. The experiment was performed in a similar manner by trapping a single 10 µm polystyrene particle. The flow-rate was increased until the particle was lost from the trap and the corresponding drag force was calculated for the last flow-rate at which the particle was trapped.

The result, seen in Figure 53, shows a rather large deviation of the measured values from the expected quadratic curve fit. The calculated forces range from a couple of hundred pN to over a thousand. This is comparable to the trapping platform used earlier with a trapping force of roughly 400 pN at 7 Vpp.

![Figure 53: A force calibration for the circular cavity for different voltages. The forces were calculated from Stoke's drag force on a single trapped 10 µm polystyrene particle.](image)

The large deviation from the expected quadratic curve-fit can probably be explained by the fact that the forces are only acting on the particle in two dimensions. The particle can thus position itself in many different positions along the depth of the cavity and experience different fluidic drag forces at each position. In order to perform a better force calibration a 3D-focusing device would be needed.
The silicon resonance cavities are fabricated using DRIE, which is one of the main drawbacks of the technique. DRIE is a technically difficult process that will make the chips relatively expensive. Silicon as a material has been used for many decades and is well-known. However, its price may make it unsuitable as a single-use chip. Similar structures created with other techniques in other materials may be possible and thus prove the concept useful.

6.10.2 Rectangular glass capillaries for trapping

Rectangular glass capillaries are commercially available in a range of different sizes and dimensions suitable for performing acoustic manipulation in the MHz-regime. The big advantage of using commercially available capillaries is the minimal fabrication and assembly needed. However, the precision in the dimensions is not as good as for components created using microfabrication techniques. The capillaries used for trapping had a width of 1 or 2 mm and an inner diameter of 100 µm. The wall thickness was 100 µm and the tolerance in the dimensions was 10%.

To be able to access the capillary through a fluidic network, a PMMA-chip was milled to fit the capillary, see Figure 54. The PMMA-chip has three inlets and three outlets to make it possible to laminate the center inlet to an arbitrary position along the channel. A piezoelectric transducer is attached to the backside of the chip and connects to the capillary through a small amount of ultrasonic gel. A hole in the PMMA above the capillary allows for optical access to the trapped clusters in the capillary and also serves to provide a good reflector surface for the standing wave set up in the channel.

![Figure 54: The rectangular 2mm wide glass capillary attached to a fluidic chip in PMMA.](image)

Since the glass capillaries have an inner diameter of 100 µm, the expected resonance is at 7.4 MHz in water. A 10 MHz transducer, of the same kind used in the nearfield trapping platform, was used to actuate the channels. Both a 5 mm, un-diced, transducer and the diced 920 x 920 µm² transducers were tested. All transducers were mounted on a PCB and cast in epoxy.

A trapped cluster of 5 µm polyamide particles can be seen in Figure 55 and Figure 56. In Figure 55, the larger transducer is used, which seem to lead to more elongated clusters. The smaller transducers were used in Figure 56 and collected the particles in a
more spatially confined form over the transducer. The trapping strength seemed to be much higher with the smaller transducer which might be explained by the larger pressure gradient at the edges of the smaller transducer.

**Figure 55:** A trapped cluster of polyamide particles in the 2 mm glass capillary. The capillary was actuated using a 5 mm transducer.

**Figure 56:** Trapped 5 µm polyamide particles in the 2 mm capillary using a miniature transducer (seen as a square in the background).

The PMMA-system with a glass capillary as the trapping zone offers a mostly polymeric system that can be fabricated by injection-molding or other fast mass-fabrication techniques. Glue was used to bond the system into a sealed chip, but a permanent fusion bonding can be performed as well. The assembly of the system is fast and fairly simple and the cost of the chip can be kept low.
It was also noted that the lateral resonances in the glass capillary were very strong and could focus the particle suspension into different number of bands depending on the frequency used. The ability to switch between different resonant modes can be used to perform trapping in combination with focusing and separation and may be an interesting tool in the future.

6.11 Acoustophoresis in wet-etched glass chips

The work with etched glass channels and trapping in square glass capillaries triggered further investigations in the resonant behavior in isotropically etched glass channels. When scanning for the optimal trapping frequency in the glass capillaries, several lateral modes focused the particles into nice bands. It was a bit surprising that the lateral modes seem to be so strong considering that the side-walls of the capillary have an essentially isotropic profile. Other bonded glass chips aimed purely at microfluidics were tested and showed the same affinity for supporting lateral resonance modes.

Isotropically etched glass chips for continuous acoustic separation are an attractive alternative to the silicon chips used today. Silicon is a fairly expensive material and the processing methods are much more complicated and use more expensive instrumentation than the methods used to process glass. If the glass chips perform equally well as the silicon chips in a continuous flow separation, the acoustic separation methods might become more widely available within the microfluidic community.

To measure the performance of the glass chip, a design aimed at producing a single lateral node at 2 MHz was etched, see Figure 57. The width of the channel was 375 µm at its widest part and the depth was 125 µm. The design is similar to the silicon chips used for continuous flow separations at the department [70, 93, 98, 137]. The glass chip was able to focus a continuous flow of particles into a single node at low voltages and to properly characterize the chip, the work presented in paper VI was performed.

![Figure 57: The 2 MHz glass chip (A) used an external 2 MHz actuator applied to the back-side of the chip as seen in (B). The silicon chip (C) was actuated in the same manner.](image)

The single-node glass chip has a radically different cross-section than the silicon chip used. It was supposed to be isotropic, since the first glass chips tested were shown to have an isotropic cross-section. However, the single-node chip turned out to have slightly tapered sidewalls, see Figure 58.
Figure 58: SEM-images of the cross-section of the unbonded 2 MHz glass chip (a) and the anisotropic silicon chip (b).

The reason to the non-isotropic cross-section is likely inadequate mixing of the etchant. The underetch created during the etching will create a sealed-off area with low convective exchange where rest-products of the etching-process may create a more aggressive local etch. A 1 MHz channel, with a twice as wide channel, was etched in the same manner as the 2 MHz chip showed a typical isotropic cross-section, thus indicating that the tapered side-walls in the 2 MHz channel was not due to poor adhesion between the glass and the masking material.

Since silicon chips with parallel walls have been predominantly used in acoustic separation devices, the separation efficiency of the glass chip was compared to a 2 MHz anisotropic silicon chip with straight side-walls. The chip design used can be seen in Figure 59 together with a schematic overview of the separation procedure used.
To compare the two materials, a suspension of polyamide particles was continuously infused into the chip and focused into the center outlet. The separation was performed for different flow-rates and particle concentrations. The acoustic input was kept at 0.5 W during all separations. The number of particles in each of the three outlets was counted with a Beckman Coulter Multisizer 3, and the ratio of the particles in the side-outlets and the particles in the center outlet was used as a measure of the separation efficiency. The separation efficiency was measured for different flow velocities and particle concentrations and the results can be seen in Figure 60 and Figure 61.

For a direct comparison between the glass and silicon chip, the flow velocity was used instead of the flow-rate. The flow velocities of the two chips were calculated through a three-dimensional COMSOL simulation. Due to the smaller cross-section of the glass chip it will experience a much higher flow-velocity for a given flow-rate when compared to the silicon chip. Since the velocity determines the time a particle spends in the force-field it is an important parameter to consider when comparing devices.
Separation efficiency vs flow rate
Particle concentration: 2%

Figure 60: The separation efficiency of the glass and silicon chips for different flow velocities and a particle concentration of 2%. The acoustic power was fixed at 0.5 W. The glass chip shows more or less the same performance as the silicon chip.

Separation efficiency vs Concentration

Figure 61: The separation efficiency for different particle concentrations. The flow-velocities were 12.9 cm/s for the glass chip and 14.8 cm/s for the silicon chip. The acoustic input was kept at 0.5 W.

The performance of the glass chip differs only slightly from the silicon chip in spite of its non-parallel side-walls. There is clearly more work needed to fully understand the resonant modes in differently shaped cavities, but the fact that wet-etched glass channels can function equally well in an acoustic particle manipulation system is exciting.
A further investigation in how different cross-sections in different materials behave would be very interesting. Would a silicon chip with a 45 or 54 degree angle wall or an isotropic silicon channel work? If, by modifying the etching process, a truly isotropic glass chip would have been used, would that have been more or less efficient or does the cross-section simply not matter?

Nevertheless, we hope that the ease of fabrication of glass chips will make the acoustic techniques available and interesting to a larger part of the research community than before.
7 CONCLUSIONS

The work in this thesis demonstrates a non-contact trapping platform for microsystems based on high-frequency ultrasonic standing waves in the near-field of a transducer. The system is shown to be capable of trapping microparticles and cells by levitating them in the center of a microfluidic channel. The microfluidic network allows for easy administering of reagents and was used to perform both particle-based bioassays and viability assays on live cells. The viability assay and the culturing of yeast cells suspended in the trap, indicate that cells are not adversely affected by the standing wave.

The trapping platform has been utilized in performing DNA-separation as a sample preparation step for rape case analysis within the field of forensics. A differential extraction of male and female DNA was performed in thirteen minutes as opposed to the several hours the standard technique would take. A fourfold enrichment of the male fraction was accomplished in this time and the microfluidic approach will facilitate the integration of this sample preparation step with the following analysis steps.

The two-dimensional array envisioned in the dynamic arraying concept remains to be implemented. Since the results show that the ultrasonic trapping concept is a powerful and versatile tool, a two-dimensional trapping array is still of interest and should be addressed in future work.

Apart from the trapping platform, an all-glass acoustic device for cell and particle separation was demonstrated. Acoustic separation chips previously reported have been manufactured in silicon. By showing the possibility to perform separations with comparable efficiency in glass devices, fabrication time and device cost can be significantly reduced.

With the continued development toward miniaturized systems, an increased need of particle handling in microsystem is expected. Due to its ease of design and high flexibility, acoustic particle manipulation has the potential to become a powerful and flexible tool within the field of microsystem technology.
8 POPULÄRVETENSKAPLIG SAMMANFATTNING


I avhandlingen beskriver utvecklingen av en metod som gör det möjligt att fånga in och hålla kvar celler på specifika platser. Metoden bygger på att låta högfrekvent ljud (ultraljud) skapa stående vågor i små kanaler. Om en cell transporterar in i en stående våg med hjälp av ett vätskeflöde kommer den att hållas kvar där så länge ultraljudet är på. När man stänger av ultraljudet kommer cellen att färdas vidare i systemet med vätskeflödet. Det finurliga med att använda sig av ultraljud är att det inte bara fångar in celler utan även får dem att sväva utan kontakt med väggarna i kanalen. Detta är en stor fördel när man vill titta på celler som normalt inte har kontakt med några ytor, till exempel röda blodkroppar som normalt bara åker runt i blodet.


Ett annat viktigt användningsområde är DNA-analys av bevis från våldtäktsfall. När man tar ett prov från offret för att säkerställa bevis får man en blandning av celler från
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REFERENCES


Trapping of microparticles in the near field of an ultrasonic transducer

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Abstract

We are investigating means of handling microparticles in microfluidic systems, in particular localized acoustic trapping of microparticles in a flow-through device. Standing ultrasonic waves were generated across a microfluidic channel by ultrasonic microtransducers integrated in one of the channel walls. Particles in a fluid passing a transducer were drawn to pressure minima in the acoustic field, thereby being trapped and confined at the lateral position of the transducer. The spatial distribution of trapped particles was evaluated and compared with calculated acoustic intensity distributions. The particle trapping was found to be strongly affected by near field pressure variations due to diffraction effects associated with the finite sized transducer element. Since laterally confining radiation forces are proportional to gradients in the acoustic energy density, these near field pressure variations may be used to get strong trapping forces, thus increasing the lateral trapping efficiency of the device. In the experiments, particles were successfully trapped in linear fluid flow rates up to 1 mm/s. It is anticipated that acoustic trapping using integrated transducers can be exploited in miniaturised total chemical analysis systems (μTAS), where e.g. microbeads with immobilised antibodies can be trapped in arrays and subjected to minute amounts of sample followed by a reaction, detected using fluorescence.

Keywords: Ultrasound; Near field; Trap; Piezoelectric; PZT; Microfluidic; Particles

1. Introduction

Particle handling in miniaturised total chemical analysis systems (μTAS) has begun to receive attention due to the prospect of using particles or beads with a large surface area and tailored surface chemistry for performing bioassays. Physical barriers may be utilized to trap particles during reaction and analysis [1–3] but to allow for a more flexible trapping of particles in biospecific arrays in a fluidic chamber, an alternative trapping technique is desired. In this paper we thus evaluate acoustically controlled trapping of microparticles within a microchannel using integrated ultrasonic microtransducers. The integration of microtransducers is expected to create opportunities for novel applications that are not possible to reach with conventional macro scale components, such as arrays of distributed and individually addressable particle trapping positions in microfluidic systems.

Particles subjected to acoustic waves are influenced by acoustic radiation forces, which are particularly strong in standing wave fields [4]. The forces can be divided into axial and transverse components of the primary radiation force, and secondary particle–particle interactions due to scattering of incident waves [5]. The acoustic properties of the particulate material as
compared to the surrounding medium determine whether the primary radiation force is directed towards the pressure nodes or antinodes in a standing wave. Typical polymer particles gather at pressure nodes when subjected to an acoustic standing wave in water. The magnitude of the radiation force is proportional to the acoustic frequency [5] and for particle manipulation it is therefore advantageous to increase the frequency to the ultrasonic region. Consequently ultrasonic has successfully been used to manipulate particles or biological material, e.g., as acoustic tweezers [6] and for particle separation from continuous fluid flow in macro- [7] and microscale devices [8]. Two-dimensional trapping and manipulation of microorganisms has been performed using orthogonal standing waves [9]. Size-selective ultrasonic trapping of microbeads in capillaries has also been investigated [10,11] in order to allow separation of immunocomplexes for trace-amount protein detection. Other bio-related applications making use of acoustic forces include separation of fat from blood during cardiovascular surgery [12] and the retention of mammalian cells in cell culture fermentations [13]. None of these papers however address the issue of trapping particles in microfluidic systems with the prospect of performing bioassays in a bead-based array format.

When performing bead-based bioanalytic assays within a trapping device, it is desirable to keep the particles away from the interior surfaces during trapping. This is thought to minimise carry over between assays when new beads are introduced in the trap. By designing an acoustic resonator to position a pressure node within the fluid, particles can be collected within this nodal plane. The acoustic field in resonators for ultrasonic particle manipulation has been investigated and modelled previously [7,14–16]. The modelling includes the thickness and acoustic properties of the resonator layers (transducer, matching, fluid and reflector) but is restricted to the one-dimensional case with propagation of the sound wave in one direction only.

In this paper we present a novel microfluidic device based on a microresonator that employs integrated microtransducers for particle trapping. To improve electrical matching of the piezoceramic microtransducers to the driving electronics [17], the transducers are constructed with integrated multilayer sandwich electrodes. Considering that the lateral dimensions of the transducers are in the same order of magnitude as the acoustic wavelength in the resonator, three-dimensional effects such as near field pressure variations [18] are expected. This has previously been noted to allow stable off-axis positioning of specimens in a macroscale acoustic levitator radiating in air [19]. The possibility to use near field effects for lateral particle trapping in a microfluidic device is evaluated experimentally and the results compared with calculations of the three-dimensional acoustic field within the resonator.

2. Experimental

2.1. Design

The design of the microresonator is shown schematically in Fig. 1. The \( \lambda/2 \) wavelength fluid layer (b), conducting the particle suspension was enclosed by an air-backed miniature transducer (c) with a thickness of \( \lambda/2 \) and a reflector (a) with a thickness of \( (2n+1)\lambda/4 \). The transducer was thus positioned in direct contact with the fluid.

2.2. Transducer manufacturing

The lead zirconate titanate (PZT) transducers were batch-wise fabricated in the green state using multilayer wet building of PZT \(^1\) slurry and computer numerically controlled (CNC) machining of screen-printed platinum electrodes according to previously published work [20]. Transducer elements were manufactured with green dimensions 1.0 mm square. The elements were composed of a total of three active PZT layers sandwiched between inactive layers. After sintering, external Ag electrodes were applied for electrical connection to the internal electrodes. The sintered transducer elements were measured to be 0.80 × 0.83 mm\(^2\) with a layer thickness of active layers being 36 \( \mu \)m. The transducer elements were thinned by polishing, tuning the element thickness resonance to near 10.6 MHz. Finally, the PZT material was polarised by applying 50 V over the external electrodes for 2 min at room temperature.

2.3. Device manufacturing and assembly

Printed circuit boards (PCBs), 26 × 26 mm\(^2\), supplying electrical connection to the transducer elements were CNC machined from copper–epoxy laminate. Holes were drilled through the boards for fluidic connections and in the positions of the transducer elements for air backing. On each board, three transducer elements covering the drilled holes were mounted and electrically connected using conductive Ag epoxy \(^2\) as shown in Fig. 2. Epoxy \(^3\) was thereafter cast over the boards, hardened and polished down to the upper surface of the transducer elements. Silicon rubber connectors were attached to the backside of the PCBs for fluidic connection (inlets and outlets) via the drilled holes and through the epoxy layer.

Channels were microfabricated using SU-8 \(^4\) thick photoresist on 1.55 mm thick soda-lime glass substrates. The channels were designed to be 1.0 mm wide and to fit

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1 EDO EC-76, EDO Ceramics, USA.
2 Circuit Works 2400, Chemtronics, USA.
3 Epotek 353ND, Epoxy Technology, USA.
4 SU8-50, MicroChem, USA.
the fluidic connections on the PCBs with one inlet for particle suspension, one for water and a common outlet, as shown in Fig. 3. Excess fluid connections, forming crosses at the transducer positions, were added for future use and to avoid lateral channel resonances. SU-8 was applied by spin coating, forming an approximately 80 μm thick layer that was patterned using photolithographic techniques [21]. The substrate was finally diced into 19 × 19 mm² chips, the SU-8 thickness was measured and in some cases it was adjusted by polishing. The device was assembled as illustrated in Fig. 4 and clamped to seal the channel to the transducer board and to assure for correct channel height.

In order to use the soda-lime glass plate (thickness 1.55 mm, sound velocity \(v_s = 6000\) m/s [22]) as a \(\lambda/4\) reflector, the frequency should be tuned in relation to the plate thickness: 8.7 MHz corresponds to \(9\lambda/4\), 10.6 MHz to \(11\lambda/4\) and 12.6 MHz to \(13\lambda/4\). Between these frequencies the plate have eigenfrequencies corresponding to \((2n + 1)\lambda/2\) at 9.7 MHz and 11.6 MHz. Reflector plates with SU-8 channel heights of 59 μm and 72 μm, corresponding to \(\lambda/2\) resonance in water at 12.7 MHz and 10.4 MHz respectively, were chosen for the device assembly. These frequencies do not exactly match the frequencies yielding \(\lambda/4\) in the reflector, but was the closest match feasible. The epoxy-covered transducer board and final assembled device are shown in Fig. 5.
2.4. Acoustic field distribution

The acoustic field distribution inside the resonator was calculated using the angular spectrum approach (ASA). ASA is based on the fact that if the complex scalar field distribution of a monochromatic wave is Fourier-transformed across any plane the resulting spatial Fourier components can be identified as plane waves travelling in different directions away from that plane [23]. When this plane contains the source field, understood as the field immediately in front of the source, it is designated the source decomposition plane. The field amplitude at any other point in the medium (or across any other parallel plane) can be calculated by adding the contributions of these plane waves, taking into account the phase shifts they have undergone during propagation. ASA enables an efficient analysis of the transmission and reflection from planar interfaces between different media since it formulates scalar diffraction theory in a framework similar to that used in the theory of linear, time invariant systems.

ASA appeared to be a very suitable tool for the analysis of an acoustic field inside the resonator filled with liquid. First, the field emitted by the transducer was expressed as a sum of planar waves with different angles using a two-dimensional fast Fourier transform (2D FFT). As a first approximation it was assumed that the particle velocity vector at the surface was normal to the surface and uniformly distributed, i.e. originating from an ideal piston transducer. Then, the field at the reflector plane was calculated and the wave reflected from the rigid reflector was constructed using principles of geometrical optics. The field inside the resonator was obtained as a superposition of the field emitted by the transducer element and that reflected from the reflector. Thus, two further approximations were made: the second that the internal reflections in the reflector were neglected in the calculations, and the third that the results presented were obtained for one reflection only. Due to the second approximation the resonances resulting from the finite reflector thickness are not modelled. The third approximation was introduced since only approximate pressure patterns were needed for qualitative comparisons with the experiments.

An ASA software package developed for the analysis of elastic fields in solid immersed in water was used for the calculations [24]. First, the distribution of an acoustic field emitted by the transducer element, excited with continuous wave in water (without reflector), was calculated in the parallel and normal planes to the transducer surface. Then, similar calculations were performed for the resonator with the reflector placed at the distance \( \lambda/2 \) from the transducer.

For measurements of the acoustic field distribution, single transducer elements were mounted on 5 mm diameter round PCBs using similar mounting as described above. Measurements were performed using light diffraction tomography with the transducer submerged in water, without the reflector. This technique is an optical measurement method utilizing diffraction of light in a medium with periodical pressure variations due to the propagating ultrasonic wave. The measurement system [25] combines light intensity measurements with tomography algorithms. Compared to hydrophone measurements the method has some important advantages for the applications in this paper. The method is non-perturbing, i.e. no mechanical probe is inserted into the field, making it possible to measure the near field of a miniature ultrasonic transducer. It also offers high-resolution measurements, which is important to be able to resolve the near field pattern of the used transducer elements. The light diffraction tomography system used is schematically pictured in Fig. 6. The transducer was scanned along an axis perpendicular to the laser beam. Once the scan was completed the transducer was returned to its origin, rotated and scanned again. The procedure was repeated until the transducer had been rotated 180° and the results were presented as a pressure map of a plane parallel to the transducer element surface. All measurements in this investigation were performed using 201 samples with a sample distance of 0.01 mm and 50 projections per 180°.

2.5. Device evaluation

The resonant behaviour of mounted transducer elements connected with a coaxial cable was evaluated using a spectrum analyser 5 equipped with a reflection test kit. 6 The magnitude and phase angle of the complex impedance were plotted for transducers facing air, as well as for assembled and fluid filled microfluidic devices to obtain information on the system resonances.

To evaluate particle trapping, Teflon tubing connected the inlets of the assembled device to a syringe pump delivering water and to a manually operated syringe delivering particle suspension. The particle suspension 7 was a blood phantom, mimicking human blood, consisting of 5 μm Orgasol 8 (polyamide) particles dispersed in water with additional glycerol and dextran. According to the manufacturer the speed of sound in the suspension was 1550 ± 15 m/s. The suspension was diluted with distilled water to a particle volume fraction of 16% as measured from sedimented sample. The device was waterfilled and the excess fluid connections were sealed according to Fig. 3 before injection of particles.

5 Agilent 4395A, Agilent, USA.
6 Agilent 87512A Transmission/reflection test kit, Agilent, USA.
7 EU-DFS-BMF-ver.1, Danish Phantom Service, Denmark.
8 Orgasol, Atofina, France.
The transducer element used for trapping was con-
nected to a function generator \(^9\) that delivered a 10 V p–p sine voltage signal when connected to a 50 \(\Omega\) load. Since the impedance of the transducer element varied with frequency, the peak-to-peak voltage over the element was depending on the experimental conditions. Frequencies where the acoustic influences were stronger were qualitatively identified by scanning the frequency in steps of 100 kHz, from 100 kHz to 15 MHz. The trapping behaviour of particles in the fluid suspension over the transducer element was observed and recorded through the glass examination window of the device using an optical microscope \(^{10}\) connected to a computerised frame grabber system. The trapping sequence was also recorded using a digital video camera connected

\(^9\) HP 33120A, Hewlet Packard, USA.

\(^{10}\) Nikon SMZ 800, Nikon, Japan.
to the microscope and the trapping speed was evaluated in frame-by-frame playback.

3. Results

3.1. Acoustic field distribution

The calculated acoustic field distributions in a plane parallel to the transducer surface at the distance $k/4$ are shown in Fig. 7 (a) and (b). The plots show the pressure distributions for 10 MHz without and with a reflecting surface located at the distance $k/2 = 75 \mu m$ from the transducer surface. The normal particle velocity at the transducer surface was set to 1.0 m/s and the calculated pressure was normalized with the acoustic impedance of water ($1.5 \times 10^6$ kg/(m$^2$s)). The calculated pressure distributions in a plane normal to the element at its centre without and with reflector are shown in Fig. 8(a) and (b), respectively.

The results from light diffraction tomography measurements performed 0.3 mm ($2\lambda$) from the transducer surface at 10 MHz are shown in Fig. 9 together with a calculated pressure distribution in the corresponding plane. Dark pixels in the images indicate high local acoustic pressure.

3.2. Device evaluation

A typical impedance plot of a mounted transducer facing air is shown in Fig. 10. At 10 MHz the impedance is in the order of 10 $\Omega$. The resonance spectra of empty respective water filled devices are shown in Fig. 11. The graphs show the magnitude and phase angle of the complex impedance.

The trapping of particles was evaluated using a channel height of 72 $\mu$m, corresponding to Fig. 11(b) and (d).

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The glass was assumed to have a density of 2240 kg/m$^3$ and longitudinal and transversal wave velocities of 5640 and 3280 m/s, respectively.
The evaluation was performed using frequencies near the calculated $\lambda/2$ resonance of the fluid layer, and trapping cycles were captured for frequencies where the effect on particles was found to be strong. A complete trapping cycle is shown in Fig. 14 for 9.7 MHz, including filling channel with particle suspension, activation of transducer, washing away excessive particles at a flow rate of 4 $\mu$l/min and finally release of the trapped particle cluster. The next trapping cycle shown in Fig. 15 was captured at 11.7 MHz using a flow rate of 3 $\mu$l/min. At 10.7 MHz, corresponding to the calculated $\lambda/2$ resonance in the fluid layer, the particles were apparently ordered in a plane structure over the transducer but were not trapped at a flow rate of 2 $\mu$l/min. The particle cluster formed at this frequency was in correspondence with the result shown in Fig. 13(c), received at 12.5 MHz for the 59 $\mu$m channel. Finally, a trapping sequence showing the behaviour upon release when particles are trapped near the interior surfaces of the channel wall is shown in Fig. 16. The sequence is captured for a 90 $\mu$m channel at 9.8 MHz.

Fig. 11. Magnitude (a–b) and phase (c–d) of impedance for system with empty (dotted line) respectively water filled (solid line) channel of height 59 $\mu$m (a and c), respectively 72 $\mu$m (b and d). Circles (O) on the frequency axis indicate calculated frequency of $\lambda/2$ in the fluid layer and crosses (X) frequencies corresponding to $(2n+1)\lambda/4$ in the reflector plate.

Fig. 12. Particle trapping area, corresponding to Fig. 2, as seen through the examination window showing transducer element (a) mounted using conductive epoxy (d) on PCB with copper layer (b) and milled insulation tracks (c). The pictured element is surrounded by cast and polished epoxy, which covers the copper tracks. The SU-8 (e), forming the 1.0 mm wide channel crossing on the reflector glass plate, can be seen in the corners of the images. An arrow indicates the direction of applied fluid flow.

4. Discussion

Particles passing over the transducer are found to be efficiently trapped by activation of a proper acoustic field. In general, a strong effect on particles is correlated
to the resonances in the device, with the trapping influenced by the transducers acoustical near field. The lateral pressure gradients in the near field help in getting a strong lateral trapping of particle clusters, indicating that such effects can be utilized in miniaturised systems to increase trapping efficiency.

When subjected to the acoustic field in the vicinity of the transducer the particles form clusters showing a dis-
Distinct fine structure as can be seen in Fig. 13. The size, vibration modes and driving frequency of the transducer determine the pressure distribution in the fluid above the transducer surface and therefore also the structure of the trapped particle clusters in the fluid. The near field limit \((d^2/4\lambda)\) of a \(d = 0.8\) mm diameter circular transducer radiating in water at 10 MHz is approximately 1.1 mm. The acoustic pressure distribution within the near field region is complex, as shown in Fig. 8(a), due to diffraction effects associated with the finite sized transducer element. The calculated near field pressure distribution obtained from the ASA model is shown to agree qualitatively with the structure of the particle clusters. It should be noted that the particles used in the evaluation due to acoustic properties are collected in pressure minima, i.e. in regions between the pressure maxima in Figs. 7 and 8. The results from the light diffraction tomography measurements, Fig. 9(a), are in good agreement with the calculated pressure distribution presented in Fig. 9(b), particularly considering the simplifications associated with the modelled ideal piston transducer. Beside the possibility of different vibration modes in the transducer, deviations between tomography measurements and the calculated results may be explained by individual variations in the transducer elements due to manufacturing and mounting. Some vibration modes with different resonance frequencies are seen in the impedance spectrum of the empty device, Fig. 11. The resonance near 2.4 MHz is identified as the width resonance of the transducer. The peaks observed at the upper end of the spectrum are interpreted as thickness vibrations incorporating a few smaller peaks that possibly may be explained by the asymmetrical clamping of the element due to mounting. Also the multilayer sandwich electrode design illustrated in Fig. 2 probably influences vibration modes in the transducer, and therefore its acoustic behaviour.

The trapping device was designed to obtain a pressure node located near the middle of the fluid layer by utilizing layered resonator design principles [15] (cf. Fig. 1). The presence of a nodal plane is evident in the calculated pressure distribution, Fig. 8(b), which however does not take into account multiple reflections and transmission in the various interfaces. In the experimental set-up with the 72 \(\mu\)m channel, the channel resonance in the fluid layer was intended to match the thickness resonance of the transducer. As it can be observed near 10.6 MHz in the impedance spectra shown in Fig. 11(b) and (d), the expected resonance at this frequency appear to be moved or split. This is interpreted as an analogy with the two degree of freedom (DOF) vibration absorber effect [16,26]. According to this interpretation a vibrating mechanical system consisting of two coupled parts with coincident individual resonance frequencies demonstrate a splitting of the resonance into two separate resonance peaks surrounding the common
resonance frequency. The splitting of resonances has earlier been observed in ultrasonic spectroscopy analysis of laminate structures with different layers resonating at the same frequency [27]. The measured impedance spectrum of the resonator is complicated, Fig. 11(b) and (d), making it difficult to analyse in detail, but the obtained appearance agrees reasonably well with the 2 DOF vibration absorber effect. Particle trapping using this set-up is stronger at 9.7 and 11.7 MHz as shown in Figs. 14 and 15, possibly due to such a splitting in the system resonance. Another explanation would be that the near-field pattern gets attenuated due to multiple reflections at a frequency corresponding to the resonance in the fluid layer. Further work is needed in order to analyse the behaviour in detail. Trapping and subsequent release of particles was studied to qualitatively evaluate the position of trapped particle clusters in the fluid. If the particle clusters are not kept from the interior surfaces during trapping, particles are still present within the trapping area after release by inactivation of the transducer, as exemplified in Fig. 16. This is an undesirable condition in the future application of the device. In the trapping cycles presented in Figs. 14 and 15 the released cluster leaves no traces of particles. Thus it is concluded that the particles are kept away from the channel walls by the presence of pressure minima between the transducer and the reflector.

The trapping of particle clusters in the device relies on primary transverse radiation forces, which are proportional to the gradient in the acoustic energy density [5]. The results from calculations with the inserted reflector Figs. 7(b) and 8(b) show the existence of periodic lateral pressure gradients in the middle of the channel and in the trapping sequences shown in Figs. 14 and 15 the particles are trapped in a periodic pattern corresponding to the near field pattern. Thus it is concluded that in the evaluated device the periodic variations in the near field pressure constrain the particles laterally. The forces pulling particles together laterally in a standing wave without lateral pressure gradients are weak and originate from scattering of the incident acoustic waves by surrounding particles [5]. The trapping of particle clusters, shown in Fig. 14, is performed at a volumetric flow of 4 µl/min. For a fully developed parabolic flow profile between two parallel plates the local flow rate in the channel centre is 1.5 times higher than the average flow rate as calculated from volumetric flow. In the presented set-up the flow is however widened at the channel crossing over the transducer element with a decrease in average flow rate. It is reasonable to believe that these effects can, as a first approximation, be considered to cancel each other. Thus the volumetric flow of 4 µl/min used in the trapping experiments then corresponds to a linear flow rate of about 1 mm/s in the centre of the channel above the transducer element, where particles should be trapped. Since neither absolute measurements nor quantitative simulations of local pressure amplitudes in the resonator were performed, numerical values of the acoustic radiation forces acting on particles could not be calculated. The response speed of the trap, measured as the time to establish a fully developed particle cluster, depends on the strength of forces acting on the particles and the distances needed for the particles to reach a nodal position. Since the distances between nodal positions are small due to both the small dimensions of the microchannel, and to the presence of local pressure minima in the near field of the transducer, the particle trapping is expected to be fast, as proved by the estimated response time of 200 ms.

The particles show some tendency to agglomerate under influence of the ultrasonic field, which can be seen when they move away from the trapping area upon release. This is also evident when supplying a continuous flow of particles; agglomerates of particles can break loose from the trapped cluster. The agglomeration is thought to increase the efficiency of the ultrasonic trapping as compared to single particles since the ultrasonic forces are proportional to the agglomerate volume, while viscous drag increase as a function of the exposed surface area [5]. The acoustically induced influence on particles is not limited to the area directly above the transducer element, but reaches out laterally in the channel, as shown in e.g. Fig. 14(b). However, the forces acting on particles decrease when the distance from the transducer element increases, and particles outside the main trapping area are washed away when applying fluid flow of about 1 µl/min. Thus by introducing miniature transducer elements it is possible to perform highly localized trapping of particle clusters giving way for arrays of individually controllable trapping sites within a microchannel for microbead handling.

5. Conclusions and outlook

Particle clusters consisting of 5 µm polyamide particles were successfully trapped at linear flow rates up to 1 mm/s within a microfluidic channel using acoustic forces. The trapping was performed using miniature PZT multilayer transducer elements integrated in the channel wall, and was making use of ultrasonic standing waves with near field pressure gradients for lateral confinement of the particles. The near field pressure gradients were shown to provide strong lateral trapping, and the particles were kept away from the transducer and reflector surfaces by designing the device to obtain a pressure node near the middle of the channel. The response time of particle trapping was very short; in the order of 200 ms. The integration of miniaturised transducers opens up the possibility to trap particles in an array configuration within a microfluidic chamber, with each trapping site controlled by an individual transducer.
element. Microbead handling in such an array format may provide an increase in parallelism and throughput of miniaturised biochemical analysis. Thus, devices for biochemical analysis based on the presented trapping method will be investigated in future work. As a first step a bead-based bioassay will be performed using the fabricated devices. The trapping of beads and their position in the channel will be quantified using fluorescent particles and confocal imaging. Also processing methods that would allow integration and miniaturisation of multilayered transducer elements in two-dimensional arrays will be investigated.

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References

Dynamic arraying of microbeads for bioassays in microfluidic channels

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Abstract

This paper proposes a new dynamic mode of generating bioanalytical arrays in microfluidic systems, based on ultrasonic trapping of microbeads using acoustic forces in standing waves. Trapping of microbead clusters in an array format within a flow-through device is demonstrated for the first time using a device with three integrated ultrasonic microtransducers. The lateral extension of each trapping site was essentially determined by the corresponding microtransducer dimensions, $0.8 \text{ mm} \times 0.8 \text{ mm}$. The flow-through volume was approximately 1 $\mu\text{l}$ and the trapping site volumes about 100 nl each. The strength of trapping was investigated, showing that 50% of the initially trapped beads were still trapped at a perfusion rate of 10 $\mu\text{l/min}$. A fluorescence based avidin bioassay was successfully performed on biotin-coated microbeads trapped in the flow-through device, providing a first proof of principle of the proposed dynamic arraying concept. The dynamic arraying is believed to be expandable to two dimensions, thus, with a prospect of performing targeted and highly parallel protein analysis in microfluidic devices.

Keywords: Ultrasound; Microchannel; Trapping; Bead; Array; Proteomics

1. Introduction

With the sequencing of the human genome being completed, the task of mapping the proteome has been outlined as the next major milestone in life science research. Although the global proteome mapping effort is an important goal, yet more targeted protein expression investigations, closely related to disease development are still gaining the attention from a majority of the biomedical proteomic research community [1]. A recent trend is the search for biomarkers in biofluids derived from diseased tissue or natural secrets from organs related to or involved in the disease progress [2]. It is envisioned that the identification and abundance mapping of biomacromolecules/biomarkers involved in early disease states may very well provide a new approach to clinical diagnostics and/or an efficient route to the development of new drugs and disease treatments [3,4].

In line with the discovery of new biomarkers being early indicators of disease development, the need for techniques to screen samples for such biological components will increase proportionally. Hence, the recent and very intense development in the area of protein arrays [5,6]. Although, much focus has been put on the development of global protein microarrays analogous to DNA arrays [7], protein arrays composing directed biological questions may very well provide equally important information and in a more targeted approach answer the questions related to a biological pathway or a given disease through out its progress.

In order to successfully develop protein arrays, high quality affinity probes directed against the specific proteins/biomarkers that correlate to the disease state are needed.
Thus, the development of antibody libraries has become a specific area of interest with several industrial players. Once the desired antibody library is developed and available, a technology for arraying these compounds for parallel read-out has to be presented. Today, ink-jet technology is one of the most common ways of producing two-dimensional arrays of biological compounds [7–9]. Individual antibodies can thus be deposited in an array pattern onto a flat surface, e.g. a glass slide, which is chemically modified to facilitate efficient immobilisation of antibodies [6]. These types of protein arrays are disposable, enabling a single screening of one biological sample for a given set of antibody–antigen interactions.

Bioarrays for protein identification in microfluidic systems using microbeads as the solid phase for immobilisation of antibodies have previously been performed by several groups, utilising the increased surface area, and thus the high binding capacity of the beads as compared to solid surfaces [10,11].

The use of functionalised microbeads for performing highly sensitive bioassays is an attractive approach to targeted protein analysis. In order to perform such assays in a flexible and reusable format, means of trapping and manipulating microbeads in microfluidic systems are sought. Several authors have in the past demonstrated manipulation and separation of particles or cells in fluidic systems by making use of acoustic forces in standing waves [12–15]. Acoustic separation of microparticles in micromachined devices has been reported [16]. It has been shown that cavitation, and related detrimental damage of biomaterial, can be avoided by using high frequency ultrasound above 1 MHz [17]. Thus, it is possible to use the same principle to extract erythrocytes from blood plasma and to eliminate fat particles from blood [18]. Neither of the above papers has however addressed the issue of trapping beads or particles in streaming microfluidic systems in order to perform bioassays. The possibility of trapping beads in a capillary using a focused ultrasonic transducer has been studied to enable separation and detection of trace amounts of proteins [19,20]. The trapping was performed by generating a standing wave in the length direction of the fluid flow through the capillary. A recent report from our group [21] describes the design and development of a microfluidic device with integrated piezoceramic microtransducers for localised and spatially controlled ultrasonic particle trapping. The integration of several microtransducers gives way for the creation of individually controlled trapping sites along the length of a flow channel, thereby providing means for the formation of biospecific bead cluster arrays.

This paper proposes a new dynamic mode of generating protein arrays using ultrasonic forces to trap the solid phase, making the simultaneous screening of a whole set of different biological samples possible. By performing the antibody/antigen interaction on chemically activated microbead clusters in a laminar streaming microfluidic system rather than on solid surfaces and by trapping such bead clusters of different antigen specificity (A, B, ..., X) in an array pattern using acoustic forces, one can address each bead cluster array with an individual sample stream (1, 2, ..., Y), as illustrated in Fig. 1. In this way, the solid phase (bead cluster), on which the bioassay is performed, can be disposed after completed analysis by simply switching off the bead trapping forces and displacing the beads from the analysis zone. After loading a new set of bead clusters into the array, the next assay can be performed. The concept of dynamically generated protein arrays can in principle be integrated and controlled in a closed microfluidic system that is highly amenable for automation. A core of the proposed protein array technology is the development of means for individually controlled trapping and release of microbead clusters.

The present work demonstrates a bead trapping microarray consisting of three piezoelectric microtransducers integrated in a microfluidic channel. The successful loading of an array and individual control of the bead clusters is demonstrated for the first time. The strength of the acoustic trap, i.e. loss of beads during streaming conditions at different perfusion flow rates is reported. Finally, a model bioassay using fluorescence-labelled avidin binding to biotin-coated beads is performed in the device, demonstrating successful on-line bead trapping and biochemical read-out in a flow-through format.

2. Experimental

2.1. Microarray device

The fabrication of the ultrasonic microarray device has been presented and discussed in detail in a previously published paper [21], and is thus only briefly presented herein.
The device comprises an array of three individually addressable bead-trapping sites. Each trapping site is designed as an acoustic resonator consisting of a miniature ultrasonic transducer and a reflector, enclosing a bead conducting fluid layer. The resonator is designed to essentially obtain a pressure node in the middle of the fluidic channel above the transducer, keeping the particles away from the interior channel surfaces. The function is illustrated in Fig. 2, showing a cross-section of a resonator array. Previous work [21] has shown the existence of regions with strong laterally confining acoustic forces in the near field of the transducers that facilitate bead cluster trapping, since forces acting on the particles are proportional to gradients in the acoustic energy density [22].

The evaluated device, shown in Fig. 3, consisted of a microstructured SU-8/glass channel plate clamped to a printed circuit board (PCB) carrying the transducer array as well as the electrical and fluidic connections. The piezoceramic microtransducers were batch fabricated using a multilayer thick film prototyping process [23], based on computer numerically controlled (CNC) milling. Three transducers (0.8 mm × 0.8 mm × 0.2 mm) were mounted on the PCB, electrically connected to copper conductors and covered with epoxy. The epoxy was polished down to the surface of the piezoceramic elements after hardening, yielding a plane upper surface. Microstructured fluidic channel plates connecting to the fluid inlets on the plane polished transducer board were batch fabricated by lithographic structuring of SU-8 on a soda lime glass substrate followed by dicing. External fluidic connections were made on the backside of the PCB. After polishing the piezoceramic material, the transducer elements were connected to a function generator1 through a control box, enabling fast switching of the drive signal to either element.

2.2. Microfluidic and imaging system

The microfluidic system was arranged as illustrated in Fig. 4. Beads were manually injected through 0.25 mm inner diameter polyetheretherketone (PEEK) tubing using a syringe. Distilled water (washing fluid) and sample was injected through 0.3 mm inner diameter Teflon tubing using two syringe pumps. The configuration of the microfabricated channel system is shown in the insert in Fig. 4, where beads were injected in (a), adjacent to the inlet for washing fluid (b). Beads and washing fluid were passing the transducers in the main flow channel, ending up in a common outlet (d). Sample was injected in an orthogonal flow channel with an individual inlet (c) and outlet (e), at the position of one of the three transducer elements (f). After filling the fluidic structure with water, excessive fluidic connections were sealed before injecting the beads.

The imaging was performed using a fluorescent microscope2 supplied with a mercury light source and filters yielding exciting light at 470–490 nm and detecting light at about 515 nm, adapted for fluorescein isothiocyanate (FITC). The shutter was normally closed and opened only during the image captures. Also a confocal imaging system3 was utilised for parts of the experiments.

2.3. Chemicals

Polystyrene beads4 (6.7 μm mean diameter) with immobilised biotin were used in the bead trapping experiments. Also FITC-marked melamine beads5 (5 μm mean diameter) were used in parts of the evaluation. A 23 μM solution of avidin6 conjugated with FITC was prepared by dissolving the lyophilised powder in distilled water.

2.4. Bead trapping and detection

Beads were injected in the water-filled channel, and conducted to the trapping site by a fluid flow of 3 μl/min supplied from the wash inlet. One transducer element was activated, trapping the beads. The frequency generator was set at delivering a 10.8 MHz, 10 V p-p sinusoidal signal. The strength of trapping was evaluated by imaging the bead loss under increasing fluid flow. The fluid flow was increased in steps of 2 μl/min until no beads were visible in the region over the transducer element. To deal with stray light, a reference image of the empty trap was captured and the intensity in each point was subtracted from captured images.

The possibility of moving beads between the three trapping sites was studied by sequential trapping. Due to induced fluorescence in the epoxy surrounding the transducer elements, more strongly fluorescent melamine beads were utilised in this experiment to allow imaging of the complete array. Beads were injected and conducted to the first trapping site using a fluid flow of 3 μl/min, which was maintained throughout the experiment. Beads were trapped by the activation of the first transducer. The driving signal was then switched to the second transducer, to which the flowing fluid

1 HP 33120A, Hewlett-Packard, USA
2 Olympus BX51WI, Japan
3 Olympus BX61WI with an FV300 confocal unit, Japan
4 PC-B-6.0, Gerlinde Kisker, Germany
5 Fluka 90641, Sigma–Aldrich Co., USA
6 Sigma A2901, Sigma–Aldrich Co., USA
conducted the beads. The procedure was repeated for the third transducer element before releasing the beads to the outlet.

2.5. Bioassay

A bead-based biotin-avidin assay was performed using one of the array trapping sites of the device. Biotin-coated beads were loaded into the trapping site and trapped as described above. The bead carrying flow was turned off, the common bead/wash outlet valve closed and the sample outlet valve opened. Avidin was perfused at 3 μl/min over the trapped beads through the sample channel and the fluorescent response was captured at time intervals of 5 s. After almost 2 min, the avidin flow was stopped, the valves switched and the washing fluid perfused at 3 μl/min for 40 s before releasing the beads. The perfusion of avidin was studied in a similar experiment repeated using an “empty” trap, i.e. without the insertion of beads. To diminish the effect of surrounding streaming and unbound avidin, the captured images of the beads were masked (using the same mask for each picture in a series), and the intensity was thereby measured over particularly stable bead clusters.

3. Results

3.1. Bead trapping

The channel height was 71 μm as measured by confocal imaging. Trapped particles in the channel were mainly positioned in the centre of the channel, 36 μm above the transducer. A small fraction of the particles was often found on the surfaces of the transducer and glass reflector. The lateral bead distribution was studied using the fluorescence microscope. A typical lateral bead distribution is shown in Fig. 5, where fluorescent beads are trapped in clusters in the fluid over the transducer element. Upon increasing fluid flow, bead clusters were pulled away from the trapping site (Fig. 6) with the measured relative bead area versus fluid flow plotted in Fig. 7.

The trapping of bead clusters by sequential activation of the three trapping sites is shown in Fig. 8. Due to exposure setting of the microscope, the beads appear smeared out when
3.2. Bioassay

The fluorescent intensity read-out of the bioassay over selected bead cluster areas is plotted in Fig. 9 (a). A similar fluorescence measurement sequence of avidin flowing into an “empty” trap, i.e. without trapped beads, is plotted in Fig. 9 (b).

4. Discussion

In order to enable fast switching of bead clusters and to minimise carry over from one bioassay to the next, it is essential to optimise the clearance of beads between subsequent assays. Thus, the bead clusters should preferably be trapped in the centre of the flow channel as this location has the highest flow and as the beads have no physical contact with the channel wall. The design of the microresonator with integrated miniature transducer elements in direct contact with the thin bead-conducting fluid layer has been shown to generate a pressure distribution with essentially a pressure node in the centre of the channel, together with periodic lateral pressure variations due to near field effects [21]. The measurements using confocal imaging confirmed that the beads were trapped in the middle of the channel, but also showed that a small fraction of beads were present at the transducer and reflector surface. This can also be seen in Fig. 8 where some beads were stuck at the middle trapping site during sequential trapping. It is anticipated that the position of the beads can be adjusted to be completely kept away from the walls by optimised driving conditions, and that improvements in transducer manufacturing will diminish individual variations between array elements. Also, by a proper selection of buffer systems, surface adherence can be substantially suppressed. The effect of the acoustic near field on the trapping behaviour is identified in Figs. 5, 6 and 8, where beads are shown to gather in clusters above a single transducer element. This implies that the distribution of beads in the trap will be influenced by the bead injection procedure. In the present device, beads were injected using a syringe and transported to the trapping site using flow from the wash inlet. Upon reaching the trapping site, the transducer was activated and beads were trapped. Beads were then sometimes still being supplied from the bead-carrying flow, giving a higher concentration of trapped beads to the left in the bead trap, as in Fig. 6 (a). This also explains the trapping behaviour during sequential trapping. In the bead trapping sequence, the drive signal was switched to the next array element instantaneously, and beads were therefore trapped upon entering the trapping site from the left. The sequential trapping series shown in Fig. 8 indicated that few beads were lost when switching between different trapping sites. Beads released from one transducer element passed in the direct vicinity of the next element due
The measurements on the strength of acoustic trapping shown in Fig. 7 indicate an almost linear relationship of remaining trapped beads versus volumetric flow. Beads were drawn from the trapping site by increasing viscous forces due to increased fluid flow. When studying individual sequences of the trapped bead clusters (Fig. 6), it can be noted that the beads mainly were removed from the trap cluster by cluster, which gave a step-wise decrease in bead area for some measurements (Fig. 7). The clusters that were trapped at the highest flow speeds were most often confined over the centre of the transducer element indicating that trapping forces were stronger there. The measurements showed that the volumetric flow could be increased up to 20 μl/min (mean linear flow rate ≈ 4.7 mm/s) before all beads were removed from the acoustic trap, and that about 50% of the initially trapped beads were still trapped at a flow of 10 μl/min. Twenty microliters per minute corresponds to displacing the volume confined within the trapping site (about 100 nl) in 0.3 s.

The results from the bioassay show that avidin had bound to the biotin-coated beads, which can be seen as a measured increase in fluorescent response marked as Δ in Fig. 9 (a). About 40 s was needed to reach a steady state in concentration of avidin over the trapping site, as seen in Fig. 9 (b). This is probably due to a gradient in concentration of avidin in the supplying sample channel when starting the assay and to the parabolic flow profile within the channel. An optimisation of the fluidics with respect of sample delivery is expected to improve the time needed for performing this type of assay. Microfluidic techniques are in general considered to yield fast chemical reactions due to the short diffusion paths in small confined volumes. The flow-through volume of the device was in the order of 1 μl, depending on the channel used, and the active volume in a channel crossing was less than 100 nl. The detected intensity at t = 0 in Fig. 9 (a) may be caused by autofluorescence of bead material, contaminations of diffused avidin or scattering of light from the fluorescent epoxy surroundings. The minimisation of this background intensity will be addressed in future work, by considering...
materials, design and detection schemes. In this proof-of-principle work, we have not yet raised the issue of detection limit using the proposed dynamic arraying technique. The concentration of avidin in the sample was in the micromolar region, and the technique is thought to enable the detection of much lower concentrations.

5. Conclusions

A new dynamic mode of generating bioanalytical arrays is proposed. Successful non-contact trapping of chemically activated microbead clusters in a microfluidic device has been presented. Bead clusters were trapped at individually controlled trapping sites using acoustic radiation forces originating from ultrasonic microtransducers integrated in the flow-through device. Studies on the strength of trapping showed that the flow could be increased to 20 μl/min (mean linear flow rate ≈ 4.7 mm/s) before all beads were pulled away from the trap by viscous forces. At a flow rate of 10 μl/min, about 50% of the beads were still trapped in their specific location. It is concluded that near field effects in the miniaturised system helped to provide strong lateral trapping forces, allowing bead trapping at considerably high perfusion rates. Performing a model bead-based bioassay using the device showed the prospect of using the technology. Biotin-coated beads were injected and trapped followed by perfusion of FITC-tagged avidin. The detection of avidin binding to the microbeads was successful. Future developments will focus on expansion of the arraying concept to two-dimensional ultrasonic transducer arrays including more detailed analysis on bead handling and cross contamination issues. Eventually, the development will enable on-line analysis of, e.g. proteins performed on biospecific bead arrays in a flow-through device.

Acknowledgements

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References


Biographies

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Urban Simu, born in 1971, graduated in 1997 with a M.Sc. in engineering physics and received his PhD degree in Engineering Science in 2002 at Uppsala University, Sweden. He belongs to the micro actuator group at the Department of Engineering Sciences, Uppsala University, as a researcher. His research interests are in processing of miniature components and actuation thereof using various functional materials. His main focus has been piezoceramic actuators for microrobotics and microfluidics.

Stefan Johansson received his PhD degree in Materials Science at Uppsala University in 1988. In 1994, he became associate professor (docent) and, in 2000, professor, both in Materials Science at Uppsala University. As one of the Swedish pioneers in micro-mechanics he has been involved in micro-fabrication and micro-robotics research for close to 20 years. During the last 10 years his research has been focused on actuation in micro and miniature systems and he is heading the micro actuator group at the Department of Engineering Sciences at Uppsala University. He is author, or co-author, of more than 80 international research papers and he, together with industrial and academic partners, have filed more than 20 patents, of which a large part is already issued. In 1996 and 1997 he and a colleague received several prestigious national innovation awards. As a consequence they founded the spin-off company, Piezomotor AB, which today develops and produces miniature piezoelectric motors for industrial applications.

Monica Almqvist received her PhD degree in Electrical Measurements in 1999 and currently holds a position as research assistant at the Department of Electrical Measurements, Lund Institute of Technology in 2000. Her research has been focused on optical measurements of ultrasound fields, airborne ultrasound and ultrasound vector doppler tomography. The past years her main interest has been in two newly started projects: developing capacitive micromachined ultrasound transducers and ultrasonic trapping in microfluidic bioanalysis systems.

Johan Nilsson obtained his PhD in 1993 in Electrical Measurements on the topic Ink Jet and Droplet Technology at the Department of Electrical Measurements, Lund University, Sweden. Following the PhD, he got a post-doc employment at the same department where he headed the research in droplet formation characterizations and silicon nozzle development. The topic for the research has since 1997 been microfluidics and microstructures with a focus on microsystems for protein analysis using mass spectrometry. He currently holds a position as Associate Professor at the Department of Electrical Measurements.

Thomas Laurell received his PhD degree in Electrical Measurements in 1995. He obtained a position as an Associate professor in 1998 and was later appointed Professor in Medical and Chemical Microsensors at the Department of Electrical Measurements, Lund Institute of Technology in 2000. Laurell currently leads the microtechnology and nanoproteomics group at the department. His research focuses on nanobiotechnology and Lab-On-A-Chip technologies, e.g., microstructured components for biomedical monitoring/analysis, implantable neural electrodes and micro components for liquid/cell sample handling in chemical microsystems, i.e., dispensing, injecting, and sampling of picoliter volumes as well as acoustic manipulation of particles and cells in microfluidic systems. In the past years a strong focus has been put on new miniaturised technology for nanoproteomics and mass spectrometry, aiming at accurate picoliter sample handling and preparation for submicroliter proteomic analysis of low abundant proteins. Also, new approaches to high density, high sensitivity protein microarrays and chip integrated microbioassays has a strong focus. For these developments the Laurell group recently set-up a lab devoted to microfluidics and nanoproteomics developments.
Characterization of Micromachined Ultrasonic Transducers Using Light Diffraction Tomography

Monica Almqvist, Member, IEEE, Marcus Törndahl, Member, IEEE, Mikael Nilsson, and Tobias Lilliehorn

Abstract—This paper demonstrates that light diffraction tomography can be used to measure the acoustic field of micromachined ultrasonic transducers (MUT) in cases in which standard methods like hydrophone and microphone measurements fail. Two types of MUTs have been characterized with the method, one air-coupled capacitive MUT (cMUT) and one waterloaded continuous wave (CW) multilayer lead zirconate titanate (PZT) transducer. Light diffraction tomography is an ultrasound measurement method with some special characteristics. Based on the interaction of light and ultrasound, it combines light intensity measurements with tomography algorithms to produce a measurement system. The method offers nonperturbing pressure measurements with high spatial resolution. It has been shown that, under certain circumstances, light diffraction tomography can be used as an absolute pressure measurement method with accuracy in the order of 10% in water and 13% in air. The results show that air-coupled cMUTs in the frequency range of about 1 MHz as well as the extreme near field of a miniaturized CW 10 MHz water-loaded transducer were successfully characterized with light diffraction tomography.

I. INTRODUCTION

The use of micromachining to develop new types of ultrasonic transducers has opened up for numerous new applications of ultrasound [1]–[6]. One advantage with this fabrication technique is the possibility to increase the frequency and bandwidth for air-coupled transducers [7], another is the possibility to integrate the transducer element with electronic circuits and physical systems [8], [9].

The increasing interest in using these transducers for varying applications also has increased the demand of new and better characterization methods. For example, there is a lack of commercial high frequency microphones working in the megahertz region. Another difficult situation is encountered when the extreme near field close to the transducer surface is to be characterized. The problem is caused by crosstalk and multiple reflections between the hydrophone and transducer surface. These situations have been addressed within two different case studies reported herein. In the first study, a capacitive micromachined ultrasonic transducer (cMUT) for air applications was developed and its characteristics were compared with a similar piezoelectric transducer [7]. In the second study, miniaturized continuous wave (CW) piezoelectric transducers were integrated in a microfluidic system for trapping of microparticles [8]. The extreme near field was used for the trapping and a characterization of the acoustic field was necessary to understand the trapping behavior.

Light diffraction tomography is an optical method for ultrasound field characterization. It was introduced by Reibold and Molkenstruck [10] in 1984. Based on the interaction of light and ultrasound, it combines light intensity measurements with tomography algorithms to produce a measurement system. Such a system has been developed by the group [11]–[13] and is used as a complement to hydrophone and microphone measurements. The method offers nonperturbing pressure and phase measurements with high spatial resolution. It has been shown that, under certain circumstances, light diffraction tomography can be used as an absolute acoustic pressure measurement method with an accuracy in the order of 10% in water [13] and 13% in air [14]. These advantages have been exploited to do high spatial resolution near field measurements of water-loaded ultrasound transducers [11], [15] and characterization of high-frequency, air-coupled transducers [14].

In this paper we show that light diffraction tomography can be used to measure the acoustic field of MUTs in cases in which standard methods such as hydrophone and microphone measurements fail. Two types of MUTs were characterized with the method.

II. LIGHT DIFFRACTION TOMOGRAPHY

A. Setup

Light diffraction tomography is based on the Raman-Nath equations describing the interaction of light and ultrasound [10]. The used setup is shown in Fig. 1 and the equipment is listed in Table I. A pressure change induced by the transducer influences the index of refraction in water (or air), e.g., when a laser beam intersects the ultrasound field the beam will be diffracted and split into several beams propagating in different directions (diffraction orders). The ultrasound field acts like a phase grating. The signal frequency and light intensity in the different diffraction orders contain information about the ultrasound frequency and the integrated ultrasound pressure along the
light/ultrasound interaction path. A lens is used to focus the diffracted light, and all diffraction orders are blocked except the zeroth and the first negative orders. This is done by the spatial filter, which is positioned in the lens focal plane. The remaining light intensity is measured by a photodetector positioned in the image plane of the optical system. This signal depends on the pressure amplitude and, with tomographic measurements and theoretical calculations, it is possible to estimate the pressure distribution in the plane in which light and ultrasound intersect. The basic theory behind these calculations is described in several papers, e.g., [10], [13], [14].

### B. Benefits

The most important advantages with light diffraction tomography can be summarized with:

- it is nonperturbing, i.e., no mechanical probe is inserted into the ultrasound field;
- theoretically, it is an absolute measurement method;
- it offers possibilities to make high spatial resolution measurements;
- it can characterize ultrasound fields in both water and air.

### C. Spatial Resolution

The spatial resolution in the reconstructed images depends mainly on the detection width, the pinhole size, the distance between two measure positions, and the number of projections/180° [15], [16]. The diameter of the pinhole has been 5–100 μm, depending on the ultrasound frequency. The system is able to resolve complex near field pattern in which pressure peaks separated 160 μm were separated. The estimated limit of the system is a resolution in the order of 20 μm. However, these measurements are very time consuming.

### D. Measurement Time

The choice of number of projections and samples is always a compromise between time and resolution. The measurement time using 501 samples and 50 projections/180° is approximately 8 hours with the used setup.

### E. Frequency

The light diffraction technique provides an almost flat frequency response as long as the Raman-Nath parameter is below 0.4 and the measurements are performed within the Raman-Nath region [17]. This is true for small transducers at low pressure levels, which often is the case for air applications. However, it is not the case for typical, medical diagnostic transducers. If the advantage with flat frequency response is to be exploited, the excitation voltage has to be decreased to a few volts. Under these circumstances the photodetector signal accurately reflects the ultrasound waveform as the integrated signal along the light/sound interaction path.

Measurements have been performed in the frequency range 500 kHz–10 MHz in water and 40 kHz–2 MHz in air [13], [14].

### F. Sensitivity

During the development of the system, the measurements were compared with hydrophone, microphone, or pulse-echo measurements. All comparison with the present setup of the light diffraction tomography system has shown equal or better signal-to-noise ratio (SNR) than the comparing method [13], [14]. The SNR is, in most cases, much lower due to the detection of the weak signal along the interaction path.

---

**TABLE I**

**Specification of the Instrumentation Used in the Used Light Diffraction Tomography Setup.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Computer</td>
<td>A personal computer with GP-IB interface.</td>
</tr>
<tr>
<td></td>
<td>Controlling software implemented in LabVIEW (National Instruments)</td>
</tr>
<tr>
<td></td>
<td>and tomography algorithms written in standard C.</td>
</tr>
<tr>
<td>Positioning system</td>
<td>Transducer holder controlled by four stepping motors, type PF264-E1.5</td>
</tr>
<tr>
<td></td>
<td>(Oriental Motor Co. Ltd).</td>
</tr>
<tr>
<td>Laser</td>
<td>Water: 10 mW, single mode, HeNe-laser.</td>
</tr>
<tr>
<td></td>
<td>Air: 635 nm, 2 mW, diode-laser, beam size 5 x 1 mm²</td>
</tr>
<tr>
<td>Photodetector</td>
<td>Avalanche photodiode module C5460 (Hamamatsu).</td>
</tr>
<tr>
<td>Function generator</td>
<td>HP33114A (Hewlett Packard)</td>
</tr>
<tr>
<td>Digital oscilloscope</td>
<td>TDS 420 (Tektronix)</td>
</tr>
<tr>
<td>Lens</td>
<td>$F = 0.25$ m</td>
</tr>
<tr>
<td>Pinhole</td>
<td>Water: 5 μm. Air: 30 μm</td>
</tr>
<tr>
<td>Amplifier</td>
<td>Air application only. 50 W</td>
</tr>
<tr>
<td></td>
<td>(Amplifier Research)</td>
</tr>
</tbody>
</table>
lower for air transducers. The explanation for this is the difficulties to generate as high pressure levels as in water. For equivalent pressure levels, the system is theoretically about 10 times more sensitive in air as compared to water measurements. This is due to the piezo-optic constant \( p_{op} \), which relates a change of refraction index to the pressure change:

\[
p_{op} = \frac{\partial n}{\partial p}.
\]

The used values of \( p_{op} \) in this study have been \( 1.51 \cdot 10^{-10} \text{ m}^2/\text{N} \) [18] for water and \( 2.0 \cdot 10^{-9} \text{ m}^2/\text{N} \) [14] for air.

**G. Absolute Pressure Measurements**

The values in the reconstructed images are absolute pressure values. Comparing measurements with calibrated membrane hydrophone and microphone confirms this [13], [14]. The uncertainty has been estimated to be 10% in water and 13% in air.

**H. Limitations**

The theoretical considerations used for the present setup are not valid in all situations. They are valid when weak acousto-optic interaction can be assumed, i.e., when the sound field is thin enough to ignore natural diffraction effects and the sound field amplitude is small enough to ignore ray-bending effects. These conditions are theoretically defined by the Klein-Cook parameter \( Q \) and the Raman-Nath parameter \( \hat{\nu} \) [19], [20]:

\[
Q \ll 1 \text{ and } Q\hat{\nu} \ll 1,
\]

where \( Q = \frac{LK}{k_e^2} \) and \( L \) is the sound field depth, \( \hat{\nu} \) is the amplitude of the optical phase retardation (Raman-Nath parameter) and can be expressed as:

\[
\hat{\nu} = k_{e0} p_{op} P_{ave} L,
\]

where \( k_{e0} \) is the wavenumber of light, \( P_{ave} \) is the average pressure amplitude, and \( L \) is the sound field depth. Some less stringent conditions have been determined: \( Q \leq 0.5 \) [20] and \( Q \leq 0.2 \) [21]. If an uncertainty up to 10% is acceptable, \( Q \leq 2 \) can be used [22]. As an example, the values for a 10 mm diameter 3.5 MHz transducer with a peak pressure of 60 kPa are in the order of \( Q = 0.16 \) and \( \hat{\nu} = 0.5 \).

The choice of equipment, components, and distances in the setup depends on the used medium (water or air) and the transducer frequency. Consequently, experience, knowledge, and accuracy are needed to perform accurate measurements. Some examples of this are:

- The laser beam should be wide enough to cover several wavelengths of the measured ultrasound to ensure good diffraction resolution. Different lasers or use of beam expansion optics are used, depending on the ultrasound frequency.
- For good SNR it is important to separate the different diffraction orders in the spatial filter plane. The separation distance between two adjacent diffraction orders depends on the focal length of the lens as well as on the ultrasound wavelength. As the distances between ultrasound field, lens, spatial filter, and photodetector are determined by the ordinary Gaussian lens formula, this indicates that different lengths of the optical system have to be used, depending on which kind of transducer is to be measured. Longer systems are necessary for low-frequency transducers, and a short system is preferable to reduce noise caused by vibrations and dust. It can be time consuming to adjust the correct distances between the components in the system.
- The pinhole in front of the photodetector has to be much smaller than the imaged ultrasound wavelength to prevent correct measurement of the high-frequency, light-intensity signal.

**III. Transducers**

Two different micromachined ultrasonic transducers were characterized with light diffraction tomography. Transducer 1 is a cMUT for air applications [7] and Transducer 2 is a lead zirconate titanate (PZT) multilayer transducer used for trapping microparticles in microfluidic systems [8].

**A. Transducer 1**

The cMUT, Fig. 2, was fabricated using a metallized dielectric (8 \( \mu \text{m} \)) membrane placed with its insulating side against the surface of a conducting silicon backplate with small (40 * 40 \( \mu \text{m}^2 \)) etched air cavities [7]. This kind of transducer needs a bias voltage and, depending on the bias magnitude, the resonance frequency varied between 400 kHz and 1 MHz. The diameter of the transducer was 1 cm.
Fig. 3. Transducer 2 mounted for light diffraction tomography measurements (a) and microresonator build-up (b) showing reflector (1), microchannel conducting particle suspension (2), multilayer transducer element (3), baffle (4), and air backing (5). The transducer was driven by a function generator (6) to generate ultrasonic waves. An arrow indicates the flow direction within the fluid layer.

Fig. 4. Particles trapped above the transducer element (a) and a pressure map of Transducer 2 (b) generated with light diffraction tomography.

B. Transducer 2

The PZT transducer, Fig. 3(a), was fabricated in the green state using multilayer wet building of PZT slurry and computer numerically controlled (CNC) machining of screen-printed platinum electrodes [8], [23]. This is a CW 10 MHz 0.8 × 0.8 mm² square multilayer transducer integrated in a microresonator for microfluidic particle trapping. The design of the microresonator is shown schematically in Fig. 3(b). The λ/2 wavelength fluid layer (2), conducting the particle suspension was enclosed by the air-backed miniature transducer (3) with a thickness of λ/2 and a reflector (1) with a thickness of (2n + 1)λ/4. Thus, the transducer was positioned in direct contact with the fluid.

If the microchannel is filled with particle suspension when the ultrasound transducer is activated, the particles will be trapped in a pattern defined by the near-field, Fig. 4(a). To confirm that this pattern was due to the complex ultrasound near field, it was decided to investigate the possibilities to characterize the transducer. The depth of the microchannel was 72 μm, which indicated that the measurements should be performed very close to the transducer surface. Theoretical calculations and light diffraction tomography measurements at a distance 0.3 mm from the surface were performed [8].

Fig. 5. Pressure maps and corresponding sectional views from light diffraction tomography measurements of Transducer 1. The measurements were performed at (a) 10 mm, (b) 18 mm, and (c) 35 mm from the transducer surface.

IV. Results and Discussion

The results from the light diffraction tomography measurements of Transducer 2 are shown in Fig. 5. Three scans were measured at the distances 10, 18, and 35 mm from the transducer surface using 201 samples, with a sample distance of 0.2 mm, and 20 projections/180°. The excitation signal was a 10 cycle, 450 kHz, 100 Vpp burst with a bias voltage of 150 V.

The theoretical near field length is approximately 35 mm. Thus, the measurements in figure Fig. 5(a) and (b) are performed in the acoustical near field. As can be seen, the pattern is asymmetric, which indicate that the transducer surface does not transmit ultrasound uniformly. The near field pattern is very sensitive to irregularities and defects in the transducer surface [15]. Note that the absolute pressure values are in the order of 100 Pa that are common values in air applications.

The pressure distribution from Transducer 2 was measured 0.3 mm from the transducer surface. The excitation signal was a 10 Vpp sine wave, and the measurement was performed using 201 samples, with a sample distance of 0.01 mm, and 50 projections/180°. Fig. 4(b) shows the result and, as can be observed, the pressure pattern is similar to the trapping pattern in Fig. 4(a) and also corresponds to the theoretical near field calculation [8].

V. Conclusions

The aim of this paper was to show the applicability of light diffraction tomography when micromachined transducers are to be characterized. Two different transducers were investigated, representing two examples of situations in which it is difficult or even impossible to characterize the ultrasonic field with commercial methods such as hydrophones or microphones. The results show that air-coupled cMUTs in the frequency range of about 1 MHz as
well as the extreme near field of a small CW 10 MHz water-loaded transducer were successfully characterized with this method.

References


Monica Almqvist was born in Malmö, Sweden. She received her Ph.D. degree in electrical measurements in 1999 and currently holds a position as research assistant at the Department of Electrical Measurements, Lund Institute of Technology, Lund University, Lund, Sweden.

Her research has been focused on optical measurements of ultrasound fields, airborne ultrasound, and ultrasound vector Doppler tomography. The past years her main interest has been in two newly started projects: developing capacitive micromachined ultrasonic transducers (cMUTs) and ultrasonic trapping in microfluidic bioanalysis systems. She is a member of the IEEE.

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Tobias Lilliehorn was born in Linköping, Sweden. He received his M.Sc. degree in physics from Linköping University in 1998 and his Ph.D. degree in microsystems technology at Uppsala University, Uppsala, Sweden, in 2003.

He is now employed as a researcher in the micro actuator group at the Department of Engineering Sciences at Uppsala University, focusing on miniaturized piezoactuators for microfluidic applications.
Noninvasive Acoustic Cell Trapping in a Microfluidic Perfusion System for Online Bioassays

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Department of Electrical Measurements, Department of Cell and Organism Biology, and Department of Child and Adolescent Psychiatry, Lund University, Lund, Sweden, and Department of Engineering Science, Uppsala University, Uppsala, Sweden

Techniques for manipulating, separating, and trapping particles and cells are highly desired in today’s bioanalytical and biomedical field. The microfluidic chip-based acoustic noncontact trapping method earlier developed within the group now provides a flexible platform for performing cell- and particle-based assays in continuous flow microsystems. An acoustic standing wave is generated in etched glass channels (600 × 61 μm²) by miniature ultrasonic transducers (550 × 550 × 200 μm³). Particles or cells passing the transducer will be retained and levitated in the center of the channel without any contact with the channel walls. The maximum trapping force was calculated to be 430 ± 135 pN by measuring the drag force exerted on a single particle levitated in the standing wave. The temperature increase in the channel was characterized by fluorescence measurements using rhodamine B, and levels of moderate temperature increase were noted. Neural stem cells were acoustically trapped and shown to be viable after 15 min. Further evidence of the mild cell handling conditions was demonstrated as yeast cells were successfully cultured for 6 h in the acoustic trap while being perfused by the cell medium at a flowrate of 1 μL/min. The acoustic microchip method facilitates trapping of single cells as well as larger cell clusters. The noncontact mode of cell handling is especially important when studies on nonadherent cells are performed, e.g., stem cells, yeast cells, or blood cells, as mechanical stress and surface interaction are minimized. The demonstrated acoustic trapping of cells and particles enables cell- or particle-based bioassays to be performed in a continuous flow system.

The global industrial standard for large-scale cell-based assays utilizes the 96 or 384 microtiter plate format. Transparent microtiter plate well arrays enable high-throughput optical readout. A frequently raised question in regards to well-based cell assays is the relevance of this model system as compared to the corresponding biological in vivo situation. A major concern relates to the metabolic turnover of the well-based system as no continuous exchange of metabolites and nutrients prevail, unlike the in vivo situation. In regards to this, it has been expressed that it is difficult to control the microenvironment within the wells, especially for slowly growing organisms that require long incubation times. Another aspect is that the fact that microtiter plates are not ideal when working with nonadherent cells, which are better processed in a wall-less environment.

It has been demonstrated that cell assays are preferably performed using perfusion-based systems, since they ensure a stable microenvironment by providing fresh nutrients and removing metabolites. Perfusion systems have now been improved by miniaturization, which has resulted in, for instance, smaller sample volumes, higher sensitivity, faster readouts, and excellent fluid control. By moving cell assays down to the microscale, the laminar flows within the microfluidic channels provide controlled transportation of cells, analytes, and cell medium. Furthermore, the overall cell consumption can be lowered since techniques exist that enable handling of a few or single objects. Finally, microfluidic systems can be automated and are more easily integrated with further analysis steps.

However, to be able to perform assays in a perfusim system a technique for controlled trapping or holding of the cells in a flow is needed, allowing the execution of a given set of chemical and/or microfluidic unit operations. This can be performed by seeding cells on the bottom of microfluidic culture wells or channels or by using a contact method, e.g., patch-clamp. Alternatively, noncontact methods for levitating objects within a

References

microfluidic environment can be employed, which is especially advantageous for nonadherent cells. For multistep assays, either several trapping sites are required or a fluidic system that not only allows for fast fluid exchange but also for rapid transport of the particles/cells between trapping sites without losses. It is also important to be able to address each trapping site with individual fluids so as to avoid any contamination or carryover from earlier steps.

Presently, several techniques that all rely on different physical phenomena are used to concentrate and trap cells, many of which are well suited for integration in a miniaturized system. These techniques include optical tweezers, dielectrophoretic trapping, and methods based on ultrasonic fields. Optical tweezers were developed by Ashkin in the 1970s and use a focused laser beam to create forces, originating from the photon radiation pressure, sufficiently strong to trap and hold a micrometer-sized particle or cell. By calibrating the system it can be used for quantitative measurements of biological forces on a cellular level. The trapped object can be translated with extremely high precision, and by rapidly scanning the laser beam between different positions, several particles or cells can be held by a single laser beam. Although optical tweezers is a high-precision technique, it can only be used on a limited number of cells, and the position of the cell needs to be known in advance. To trap and hold larger cell clusters would demand a higher number of lasers leading to a very complex system. Care must also be taken to avoid absorption of laser light by trapped cells, since this may result in a dramatic temperature increase and cell damage. The forces acting on the trapped object are in the vicinity of 100 pN depending on the intensity of the laser beam.

Dielectrophoretic trapping utilizes a nonuniform electric field that exerts an electrostatic force on polarizable particles or cells. The particles can either be moved toward the high-intensity parts of the field, i.e., positive dielectrophoresis, or toward the low, i.e., negative dielectrophoresis. This differential reaction to a field can be used for trapping combined with separation and preconcentration. The effect of the dielectrophoretic forces is decided by the electrode shape and layout, as shown by Duschi et al. This enables a multitude of particle manipulation tasks to be performed on the same chip by defining varying electrode geometries in the channels. Although it is a very versatile technique, it requires polarization of the manipulated object.

Moreover, to design the system correctly, the frequency at which the object will experience positive or negative dielectrophoresis must also be known. There is also a risk of cell damage from the stress induced by the electrical field or Joule heating if care is not taken when designing the system. The applied forces are typically in the range of a couple of hundred pico-Newton. The ultrasonic techniques usually use an ultrasonic standing wave to create a pressure node that will attract particles or cells. As with dielectrophoresis, a cell can experience either an attractive or repellant acoustic force depending on its material parameters. This can be used either to trap objects locally over an ultrasonic transducer, concentrate them within a fluidic channel, or separate different types of objects from each other. Ultrasound has been used for many years in macroscale resonators using rather low frequencies, thus resulting in low trapping forces and without the advantage of the microfluidic networks. The effect of the acoustic pressure on cells in ultrasonic traps at moderate frequencies has also been studied intensively.

Integrating an array of miniature ultrasonic transducers in a microfluidic channel, matched for operations in the 10 MHz regime, will give localized trapping with a high trapping force compared to that of the macroscale systems commonly working at lower frequencies. Advanced fluid control by means of integrated microfluidics makes it possible to create a perfusion-based system with individually addressable trapping sites that can handle small populations of cells in a noncontact mode. The basic characteristics of such a system were described by Lillicorn and co-workers in 2005, targeting controlled trapping of microbeads and perfusion-based bioassays.

We now demonstrate that the further developed acoustic trapping platform also is capable of handling live cells. The overall system design is improved by integrating the channel and the reflector into a single glass unit. This results in high geometric precision, which is essential in a resonant system. The trapping force was calculated using a single trapped particle in a fluid flow. Accurate temperature control in the trap was accomplished by characterizing the power dissipation in relation to the transducer driving voltage. The question whether the employed acoustic field is influencing cells negatively was addressed by a viability test of trapped neural stem cells and by culturing yeast cells for 6 h.

**MATERIALS AND METHODS**

**Acoustic Forces.** When an object enters an acoustic standing wave a force will act on it, forcing it to either the pressure node or the pressure antinode depending on the material parameters. The primary radiation force \( F_{PRF} \) (see the inset in Figure 1a) acting on a compressible sphere in an acoustic standing wave field

\[
F_{PRF} = \frac{1}{2} \rho C^2 \omega^2 \sin^2 \frac{\omega t}{2} \frac{\sin \omega x}{x^2} \nabla P^2 \cdot \nabla \phi
\]
was described by King\textsuperscript{28} and Gorkov\textsuperscript{29} and can be seen in eq 1:\textsuperscript{30}

\begin{equation}
F_{\text{PRS}} = -\pi P_0^2 V \rho_0 \sin\left(\frac{4\pi z}{\lambda}\right) \left(\frac{(\rho_p - 2\rho_0)}{(2\rho_p + \rho_0)} \frac{\beta_0}{\beta_p}\right)
\end{equation}

The equation is based on a spherical particle with volume $V$, compressibility $\beta_p$ and density $\rho_p$ suspended in a fluid with compressibility $\beta_0$ and density $\rho_0$. The particle is situated in an acoustic standing wave with wavelength $\lambda$ and will experience a force that will increase with higher frequency or larger volume. Most microparticles and cell types will be forced to the pressure nodes by the primary radiation force, whereas, for instance, lipid particles will be drawn to the pressure antinodes.\textsuperscript{31} The primary radiation force can, depending on the device design, either be used to position objects or to trap them.\textsuperscript{32}

Whereas the primary radiation force will move the particles into the pressure node, see the inset in Figure 1, it does not prevent the particles from moving laterally within the nodal plane itself. Lateral forces ($F_{\text{LAT}}$), arising due to spatial variations in the pressure field and acoustic streaming,\textsuperscript{33,34} will, however, keep the particles positioned at stable positions within the nodal plane. The actual trapping position are governed by the local pressure distribution over the transducer, as shown by Lilliehorn et al.\textsuperscript{26}

An inverted 3D image showing the simulated local pressure minima over the transducer, i.e., the trapping positions, is shown in Figure 1b. There are also secondary forces,\textsuperscript{35} created by pressure waves being reflected on particles, that drive the particles to form clusters, helping to stabilize the trapped objects. Thus, the trapped particles will form patterns consisting of clusters positioned at or close to the local pressure minima. The lateral distribution of the clusters will change depending on the frequency and amount of particles being trapped.

**Acoustic Resonator Design and Fluidic Channel Fabrication.** The microfluidic device is composed of a base plate of a printed circuit board, PCB, providing electrical connection to the ultrasonic transducers. An array of three in-house-developed miniature PZT transducers,\textsuperscript{36} $550 \times 550 \times 200 \mu m^3$, is mounted on the PCB and cast in epoxy as illustrated in Figure 1. A glass...
Lid with etched microchannels, 600 \( \mu \text{m} \) wide and 61 \( \mu \text{m} \) deep, is placed on the PCB and acts as a matched acoustic reflector and provides optical access to the trapping sites. The complete assembly of the acoustic device is shown in Figure 2. Silicone tubings, 1 mm i.d., provide fluidic connections to standard 1.58 mm o.d. Teflon tubings on the back side of the PCB, allowing for particle or cell injections assisted by a pressure-driven carrier flow. Channels orthogonal to the main channel enable individual perfusion of each trapping site, see Figure 2d.

To form a standing wave with a pressure node in the center of the channel, the depth of the channel is typically designed to be a half-wavelength. The reflector should be an odd multiple of a quarter of a wavelength in order to achieve a completely reflected wave.\(^{37}\) The device used in viability and trapping experiments was designed to work at the transducer resonance of 12.4 MHz giving the fluidic channels a depth of 61 \( \mu \text{m} \) to create a \( \lambda/2 \) resonator in water and thus creating the trapping zone in the center of the channel. For the device used in the temperature measurements the drive frequency differed slightly,\(^{41}12.2 \text{ MHz} \), due to slight individual variations for transducers and channel structures.

In this paper wet etching of the channels in glass was rather used, which provided the good reproducibility and high level of dimensional control required. Thereby, long-term operation of the microfluidic acoustic trapping system was enabled, which was crucial for the cell culturing experiments performed.

**Instrumentation.** An Agilent 33120A waveform generator was used to actuate the transducers with a sinusoidal signal at 12.4 MHz and an amplitude of 7 Vpp. All cell pictures were taken with a Hamamatsu ORCA CCD on an Olympus BX51WI fluorescence microscope. The fluid was driven by a SP210IWZ syringe pump (World Precision Instruments, Inc., Sarasota, FL) using Hamilton glass syringes. The fluorescence temperature measurements were performed with an inverted Nikon TE2000 epifluorescence microscope and an SE6 Monochrome CCD camera.

**Particle Trapping Efficiency.** The trapping performance of the device with respect to particle size and trapping efficiency has been evaluated using polystyrene particles ranging in size from 0.87 to 10 \( \mu \text{m} \) in diameter.

High trapping efficiency is important when manipulating cells in samples with low abundance. In acoustic applications the trapping efficiency is essentially given by the percentage of injected cells collected in the acoustic trap. In a design where side channels are used there is always a risk of losing some cells which bypass the trapping area, due to the widening flow profile in the side-channel intersection. Therefore, by hydrodynamically focusing the sample flow at the inlet,\(^{38}\) the overall trapping efficiency can be improved by directing the cell sample over the center of the transducer.

To determine the effect of hydrodynamic focusing on the trapping efficiency, a particle suspension was infused into the chip with and without hydrodynamic focusing. The number of particles passing the transducer was manually counted while a sample, containing approximately 2000 particles, was infused at two


different flow rates. The hydrodynamically focused injection used two different sample injection flow rates, 1 and 3 μL/min, and a focusing flow of 3 μL/min. The unfocused injection used a sample injection flow of 1 and 3 μL/min and no focusing flow. The particles used were 10 μm polystyrene beads in a suspension of MilliQ water with 20% glycerol added to reduce sedimentation. The particles were injected for 30 s using the lower flow rates and for 20 s using the higher flow rate. For each flow rate the experiment was repeated six times, and the mean and standard deviation were calculated.

**Trapping Force.** In order to compare the ultrasonic trapping to other trapping techniques the lateral trapping force was measured. The lateral forces acting on the trapped objects will determine the maximum usable fluid velocities.

By relating the drag force exerted from the fluid flow to the retaining ultrasonic force a measure of the lateral trapping force was provided. A similar method was used by Tuziuti et al. showing good agreement with theory.

The drag force acting on a single spherical particle in a laminar flow is described by Stoke’s law, eq 2,

\[ F = 3\pi \mu D_p \eta \]  

(2)

where \( \mu \) denotes the fluid viscosity, \( D_p \) is the particle diameter, and \( \eta \) is the fluid velocity.

The particle Reynolds number was calculated to be around 10^−8, which is very well below the limit of 2, making the use of Stoke’s law valid.

To relate the trapping force to the drag force a very dilute particle suspension was injected using hydrodynamic focusing. A single 10 μm particle was trapped at an initial flow of 1 μL/min (0.42 mm/s) as specified by a syringe pump. After the particle was trapped the particle injection flow was switched off. The focusing flow was increased in steps of 1 μL/min until the particle was pulled away from the trapping site, i.e., when the fluidic drag force exceeded the lateral trapping force. The maximum linear flow velocity was calculated from the volume flow with the channel cross section and the parabolic flow profile taken into account. The trapping was performed at a frequency of 12.4 MHz and an amplitude of 7 Vpp. The drag force was calculated for the last fluid velocity at which the particle was still trapped and used as a measure of the lateral acoustic force on the particle.

**Temperature Measurements.** Sudden temperature changes or prolonged exposure to too low or too high temperatures may cause irreversible harm to live cells. During activation of the transducers, the piezoelectric material displays mechanical and dielectric losses which may cause a temperature rise in the fluidic channel through thermal conduction. Absorption of the acoustic energy in the fluid layer will also yield a temperature rise in the channel. The temperature increase in the fluid channel was evaluated as a function of the driving voltage at the transducer resonance frequency.

By using a temperature-dependent fluorescent dye, an accurate temperature sensing can be achieved in microfluidic systems without affecting the ultrasonic field. Rhodamine B was used due to the easy handling and high-temperature sensitivity in the interval of 0–120 °C. A calibration curve was generated by measuring the fluorescent intensity in the channel at a set of temperatures and calculating the median gray-scale intensity in each image. A linear curve fit was made in the region of 0–13 °C temperature increase relative to a reference temperature of 24.8 °C. The measured parameter is the average temperature data in the channel. To be able to account for parameters that may vary over time, such as lamp intensity, the image median were normalized to room-temperature data.

A solution of 0.1 mM rhodamine B was infused at 0.5 μL/min into the system, and the fluorescent readout was monitored during ultrasonic radiation using a fluorescent microscope (Inverted Nikon TE2000 epifluorescence microscope, Hg lamp, SE6 Monochrome CCD camera, Spot Software, 30× objectives, and 8 bit images). During calibration, a Peltier element was used to heat the chip and was mounted on the brass holder in contact with the glass channel. The device was embedded in polymer foam to minimize heat losses. A thermocouple type K (Pentronic G/G-36K) was mounted with silicone paste (Wacker silicone heat sink paste P12) in contact with the channel for temperature readings on a thermometer display (Line Seiki thermometer TC-1200, with a resolution of 0.1 °C). After activating the transducer, a stable temperature was observed for 2 min before opening the aperture and recording an image. A linear fit was made within the temperature region of 0–11 °C of temperature increase, and the standard deviation within this region was 0.3 °C.

The temperature increase in the channel due to power dissipation from the transducer can, as a first approximation, be described by Fourier’s law which assumes a linear relation between heat flow, \( G \) [W·m^−1·s^−1], and a temperature difference, \( \Delta T \), over a given distance:

\[ G = -k \frac{\Delta T}{\Delta x} \]  

(3)

where \( k \) is the thermal conductivity [J·m^−1·K^−1]. If the power dissipation from the transducer will be conductively transported through the fluid and the temperature at the reflector surface is constant, then the relation between the power dissipation and the temperature increase in the channel will be linear.

The temperature response on a voltage change is expected to be quadratic since a quadratic voltage dependence is found for the acoustic power from a transducer as well as for mechanical under the assumption that nonlinearities in the material are ignored.

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RESULTS AND DISCUSSION

Trapping Efficiency. The trapping was very weak using 0.87 μm particles, whereas 1.8 μm particles and larger were easily trapped. This corresponds well to eq 1, stating that larger particles experience a larger trapping force, making them easier to trap. The largest particle diameter possible to handle in a half-wavelength resonator is practically determined by the geometrical dimensions of the microfluidic channels.

A comparison between a hydrodynamically focused and an unfocused dye injection can be seen in Figure 3. The use of hydrodynamic focusing made it possible to direct the sample inlet over the center of the transducer. This avoids loss of particles that bypass the trapping area due to the widening flow profile in the side-channel intersection. Further, the risk of losing particles has experimentally been seen to be higher when the trapping occurs near the edges of the transducer.

Generally, the trapping efficiency is very high for all cases, see Table 1. However, an increase in the number of lost particles can be seen in the case without focusing, and at higher flow rates the advantage of the focused sample injection is more apparent. The particles not trapped at the higher flow rates passed the transducer at the side channels due to the widened flow profile.

Without the hydrodynamic focusing there is a risk that particles or cells are retained in the side channels, and in a sample with low abundance of cells it is crucial not to lose any biological material. Any cells caught in the side channels may also affect the following steps in an analysis by carryover, making the hydrodynamic focusing an important step.

Trapping Force Estimation Using Single-Particle Trapping. The resulting mean trapping force from 16 consecutive measurements was calculated to be 430 ± 135 pN, which corresponds to a maximum fluid velocity of 4.6 ± 1.4 mm/s. For each measurement a single 10 μm particle was trapped at approximately the same position over the transducer surface and held against an increasing fluid flow until it was displaced from the focus region.

### Table 1. Influence of Hydrodynamic Focusing on the Trapping Efficiency

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of particles lost with focusing</th>
<th>No. of particles lost without focusing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low flow</td>
<td>1.33 ± 1.97</td>
<td>26.8 ± 13.3</td>
</tr>
<tr>
<td>High flow</td>
<td>0.67 ± 0.82</td>
<td>62.8 ± 13.1</td>
</tr>
</tbody>
</table>

* A sample containing approximately 2000 particles was infused into the device. The number of particles passing the transducer was manually counted with and without hydrodynamic focusing. When a focused injection was used, a clear improvement was noted, which was even more pronounced at higher flow rates.

Biological Material. To investigate how cells respond to being suspended in the high-frequency ultrasonic standing wave, three cell experiments were performed.

An initial cell-trapping experiment was performed using spleen cells from rats, fluorescently marked using acridine orange.

The yeast strain, Saccharomyces cerevisiae UMR 106 (MATa LEU2-VENUS ade2-1 can1-100 his3-11,15 ura3-1 lys2-deletion), from U. Mortensen’s collection (Technical University of Denmark, Denmark) was used in the subsequent cell culturing experiment. This yeast strain has an ORF coding for yellow fluorescent protein (YFP) (Venus) attached in frame to the LEU2 gene. To achieve maximal signal strength from the yeast cells, the defined synthetic cell medium without leucin, SC-Leu, was used. The trapped cells were perfused with the cell medium at a rate of 1 μL/min, and images of the cell cluster were taken every hour during 6 h.

The cells used in the viability assay were a rat neural stem cell line from embryonic hippocampus, HbS-GFP, genetically modified to express green fluorescent protein (GFP). The cell medium used was standard phosphate-buffered saline solution (PBS) with a pH of 7.4. After 15 min of PBS perfusion, a viability marker, acridine orange, was supplied through an orthogonal side channel, testing the cell cluster for viability while still suspended in the acoustic trap. Fluorescent images were taken before and after the perfusion of the viability marker to determine whether or not it had migrated into the cells.
the transducer. The depth-wise position of each particle was given by the pressure node in the center of the channel. The lateral position of the studied particles was visually confirmed to be in the same near-field pressure node. The trapping force was calculated using eq 2 with the fluid velocity noted immediately before the bead disappeared from the trap.

The acoustic trapping force can be compared to optical tweezers, which usually works in the 100 pN range, and dielectrophoretic devices that work in the ranges of 400–500 pN. Direct comparisons between the different techniques are however not possible since the material parameters and device design strongly affect the forces.

**Temperature Measurements.** Increasing the driving voltage resulted in an accelerated temperature increase as expected, and the quadratic behavior between temperature increase and voltage corresponded well to theory, see Figure 4. At a drive voltage of 7 Vpp, at which level several transducers showed good trapping with 5 μm polystyrene particles, the temperature increase was 7.2 °C yielding an absolute temperature of 30.0 °C.

Since the temperature increase is dependent on the voltage over the transducer, it is possible to tune the channel temperature to a certain degree by changing the actuation amplitude of the transducer. This can be used to create microenvironments suitable for many cell types using the same device.

**Cell Experiments.** Three types of live cell experiments were performed to study the possibility of carrying out live cell assays. The first experiment was conducted with spleen cells from rats and was primarily aimed at trapping and holding live cells in the acoustic standing wave. The cells were trapped instantaneously as the ultrasound was activated while perfused by buffer. Part of the trapping area, showing three trapped cell clusters, can be seen in Figure 5.

Figure 5. Subsection of the trapping area showing trapped clusters of rat spleen cells that are held against a fluid flow to investigate cell-trapping behavior. The cells were fluorescently marked with acridine orange to improve visibility.

Figure 6. Growth of YFP-expressing yeast cells, UMR106, trapped in the acoustic device while being perfused with cell medium. The images show the increase of the number of cells in the cell cluster after 1–6 h of cultivation. The successful growth indicates that the cell proliferation is not affected by the high-frequency acoustic radiation. The horseshoe-shaped pattern is caused by the cells clustering in the near-field pattern.

Figure 7. Viability assay on a cluster of acoustically trapped neural stem cells, HiB5-GFP, modified to express green fluorescent protein, GFP. The cells were exposed to 15 min of continuous ultrasonic radiation and were then perfused with acridine orange through the side channels as a test for viability. In (a) the cells are shown shortly after being trapped, and in (b) the cells have been subjected to 15 min of continuous ultrasonic radiation in the trap, after which they were perfused with acridine orange. The acridine orange has migrated into the cells, indicating a viable cell cluster. The exposure time of the camera is approximately 6 times longer in (a), demonstrating the dramatic fluorescence increase of the cells after acridine orange resorption in (b). Only a very small shape and rotational change of the cell cluster can be seen after 15 min of trapping.

Analytical Chemistry, Vol. 79, No. 7, April 1, 2007
buffer flow, showing that the trapping did not occur at the channel walls. Thus, a continuously perfused noncontact cell trapping was accomplished.

In the second experiment, yeast cells expressing yellow fluorescent protein (YFP) under the control of the LEU2 gene promoter were maintained and grown in the acoustic trap for 6 h to ensure that no long-term effects resulted from the acoustic forces on live cells. The cells were continuously perfused with the cell medium to promote growth, and images were taken every hour for 6 h, see Figure 6. The successful cell growth clearly indicates that the acoustic environment does not affect the cell proliferation. Therefore, it can be anticipated that this device can be used to study proliferation of cells as a function of different media. For example, a study of the response time of yeast cells to changes in their microenvironment could be performed.

Finally, a viability test was performed on acoustically levitated neural stem cells, HiB5-GFP, see Figure 7. The cells were trapped and exposed to acoustic forces for 15 min. The viability test was performed while the cells were still suspended in the standing wave by administrating acridine orange through the side channel. The cells showed no perceptible damage from the acoustic radiation and responded well to the viability test, as indicated by the increase in fluorescence caused by the active transport of the acridine orange into the cells.

The cell experiments indicate that the ultrasonic environment within the standing wave does no perceptible harm to live cells. Future work will include longer acoustic exposure combined with the exposure to different media and analysis of the mutability effect.

CONCLUSIONS

Noncontact trapping and retention of cells in microfluidic networks by means of acoustic standing wave forces are demonstrated as a platform for perfusion-based cell handling and assay. The current improvements of the described acoustic trapping microfluidic platform provide stable resonator dimensions over extended periods of operation. A key feature is the manufacturing of the microfluidic channels directly in the glass reflector layer, thus avoiding the use of photolithography-defined polymer gaskets that may undergo swelling during the experiments. This now enables longer periods of stable operation of the noncontact acoustic particle/cell trapping platform as demonstrated in the cell culturing experiment over 6 h. The temperature measurements indicate that the thermal environment in the acoustic trap is at a level where no negative effects are expected on cell behavior. The viability tests verify that the acoustic intensities used give no indication of being harmful to the cells, but this will have to be confirmed by extended studies, including gene expression profile analysis.

In future work, the possibility of single-cell trapping will enable the study of growth and response of single cells under the influence of various environmental conditions as set by the perfusing fluids. We are currently targeting further studies in noncontact microfluidically controlled cell-based assays. The upscaling to multiple trapping sites in an array format will further provide the ability to either generate statistical data on multiple identical experiments or the possibility to run screening protocols with concentration gradients of a given substance. It will also enable efficient analysis of the cell response to different substances from a library. A further outlook is to link this cell-based trapping and chemostimulation platform to the nanoproteomic chip technologies developed earlier in our group, enabling differential protein expression mapping of small cell populations due to the integrated and confined microfluidic format.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

A video sequence of trapping of hydrodynamically focused fluorescent microparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Acoustic Differential Extraction for Forensic Analysis of Sexual Assault Evidence

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Keywords: acoustic trapping, epithelial cell, sperm cell, differential extraction, forensic

INTRODUCTION

Forensic DNA analysis is of penultimate importance in our criminal justice system but manacled by the time-consuming, labor-intensive nature of the analytical process. As a result, most forensic laboratories suffer from a significant backlog of casework awaiting DNA analysis. In 1999, the National Institute of Justice estimated the backlog of sexual assault evidence to be approximately 500,000 cases¹. As a result, thousands of sexual offenders that could potentially be proven guilty with DNA evidence are not challenged by the justice system simply because the evidence remains unanalyzed. Therefore, the forensic community stands to benefit significantly from any technological advance that improves the analytical speed and throughput in a manner conducive to automated analysis of sexual assault evidence for forensic DNA profiling. Efforts are underway in a number of laboratories to develop integrated microfluidic devices to carry out DNA profiling², ³. In the most functional embodiment, these will possess the ability to carry out all of the necessary sample processing steps – cell capture, DNA extraction and PCR – along with the STR profile generation from evidentiary material. The focus of this report is a sample processing step unique to sexual assault evidence: differential extraction⁴.

The primary form of sexual assault evidence, vaginal swabs, requires differential extraction to separate the male and female fractions of DNA in the sample. This method exploits the susceptibility of epithelial cells (primarily from the vaginal lining of the female) to nuclear membrane lysis when exposed to a ‘mild’ detergent. In contrast, sperm cells, with a highly disulfide-crosslinked nuclear membrane, are resistant to lysis until the disulfide bonds are reduced, at which point the nuclear membrane can be lysed and the DNA released. The mild lysis buffer not only serves to lyse the epithelial cells, it is key to desorbing the biological material from the vaginal swab, yielding an epithelial cell lysate (incl. free DNA from the female) and sperm cells. With sexual assault evidence, it is not unusual for the sperm tails to have been proteolytically digested from the heads in the mild lysis buffer due to the proteinase K added to the buffer. In the conventional method used today, multiple centrifugation and wash steps are performed and the sperm cells (pelleted) and the female DNA (supernatant) are separated into fractions from which DNA can be extracted and purified. From the purified male and female DNA, short tandem repeat (STR) fragments can be amplified separately in order to obtain a DNA STR profile from both the victim and perpetrator.

Poor efficiency of separation of the male and female fractions in DE can lead to STR profiles that exhibit a mixture (i.e., alleles from multiple individuals) and make interpretation difficult if not impossible. Even more problematic is the fact that the DNA profile from the perpetrator is often not observable in the presence of overwhelming amounts of female DNA. For this reason, a highly-purified fraction of sperm cells (or male DNA) is not only advantageous to forensic laboratories, but may also enable an analyst to obtain a perpetrator’s DNA profile where one is otherwise unable to do so.
The conventional method currently utilized in forensic labs suffers from several disadvantages. Despite the number of wash steps implemented in the conventional differential extraction method, the sperm cell fraction is often of suboptimal purity, contaminated with a significant amount of female DNA (up to 30% of the total DNA), complicating the interpretation of the resultant STR profile. While no detailed study can be found in the literature addressing the carryover of female DNA into the male fraction using the accepted conventional method, these observations appear to be commonplace in forensic laboratories and have been suggested to occur in 40% of samples. Consequently, there is a dire need to improve this process in a way that improves the purity of the male fraction and, at the same time, enhance the overall speed of analysis.

Efforts are being made to move the forensic analysis to a miniaturized platform as a step in developing a totally-integrated, fully-automated microfluidic device for forensic DNA analysis. Providing such technology to forensic laboratories will enable them to address the continual influx of casework awaiting analysis in a timely manner, as well as the backlog of DNA evidence. In addition, there is an inherent advantage to the microchip format, for both the forensic and clinical communities, in that it is a self-contained analysis system. That is, decreasing the amount of manual sample manipulation (number of tube transfers) greatly diminishes the potential for loss of material or contamination of the sample. In a forensic laboratory, any steps to prevent contamination and/or loss of sample are greatly valued. Thus, a miniaturized assay will be of great value to the forensic community as a stand-alone device as well as its potential for integration with additional sample-processing steps on a single microdevice.

The method presented here, termed Acoustic Differential Extraction (ADE), utilizes non-contact acoustic forces in a valveless microfluidic device to trap sperm cells from of a biological mixture obtained from sexual assault evidence (vaginal swab), as a means of obtaining highly-enriched fractions of male and female DNA for downstream forensic DNA analysis. The advantages of such a mechanism for isolating the male fraction include a concentrating effect (large number of sperm in a nanoliter volume) and minimizing the inadvertent contamination of female epithelial cell DNA.
METHODS

Acoustic trapping device

An ever-growing literature base supports the use of microfluidic methods for cell selection, enrichment, and/or capture, including partitioning, dielectrophoresis, fluorescence-activated cell sorting, immuno-specific cell capture. While valuable for select applications, each of these is associated with inherent drawbacks that preclude extension to the analysis of forensic sexual assault evidence. In contrast, the advantages of using acoustic forces in resonant chambers to separate and enrich cells/particles for forensic applications are ideal in many respects.

The use of acoustic forces for manipulating cells has been reported by several groups. Hawkes et al. demonstrated an acoustic device used for enriching yeast cells, while Nilsson and Petersson have manipulated red blood cells in mediums that allow for separation from lipid particles. Hultström et al. studied the proliferation rate on cells exposed to an ultrasonic standing wave. No direct or delayed adverse effects could be found. An acoustic device for trapping cells was developed by Spengler et al. and has been used to study the physical environment of cells in an acoustic standing wave by Bazou et al.

The acoustic cell and particle manipulation techniques are based on that an object entering an acoustic standing wave will experience a force acting upon it. The force will direct the object either to the node or the anti-node of the wave, depending on the material parameters of the object and the surrounding fluid. The forces acting on the object can be divided into primary, lateral, and secondary forces. The primary and lateral forces arise from the acoustic pressure field, whereas the secondary forces originate from pressure waves reflected from other objects. The equation describing the primary radiation force on a compressible sphere was described by King and Gorkov and is given by Eqn. 1

\[
F = \frac{-\pi \cdot P_0^2 \cdot V \cdot \beta_0}{2\lambda} \cdot \sin \left( \frac{4\pi \cdot z}{\lambda} \right) \left( \frac{5\rho_p - 2\rho_0}{2\rho_p + \rho_0} - \frac{\beta_p}{\beta_0} \right)
\]

The force is dependent on the wavelength (\(\lambda\)), the volume of the object (\(V\)), the pressure amplitude (\(P_0\)), the compressibility of the fluid (\(\beta_0\)), and object (\(\beta_p\)), and the density of the fluid (\(\rho\)) and object (\(\rho_p\)). According to Eqn. 1, the force will be stronger for larger volumes or higher frequencies. In addition, the compressibility and density of both the fluid and particles will determine if the objects will be directed to the pressure nodes or anti-nodes. Most microparticles and cells will be drawn to the pressure nodes, while other objects, e.g., lipid particles, will be drawn to the pressure anti-nodes. The lateral and secondary forces will act as centering forces, causing the objects to form clusters centered in the sound axis. The ADE method described here uses the volume difference between sperm cells and free DNA to create a force strong enough to retain the sperm cells while allowing the free DNA to pass through the trap unaffected.

The ADE device is based on a design first used by Lilliehorn et al. for microparticle trapping and bio-assays, and was later modified by Evander et al. for use in cell-assays. The microfluidic device uses an array of three miniature lead zirconate titanate (PZT) transducers, 930 x 930 µm, created by dicing a commercial piezoelectric disc (PZ26, Ferroperm Piezoceramics A/S, Kvistgaard, Denmark). The transducers are mounted on a Printed Circuit Board (PCB), and cast in epoxy (EPOTEK 301-2) (Figure 1). The epoxy layer is polished and planed and a silver layer is evaporated as a top electrode for the piezoelectric transducer. The PCB provides fluidic connections from the back-side using silicone tubing and electrical connections to the transducers by wiring soldered to the PCB. A borosilicate glass lid with wet-etched microfluidic channels is
positioned over the transducers, thereby defining the fluidic network of the chip. The glass lid is sealed to the PCB using ultrasonic gel and the entire assembly is sealed by a brass fixture (Figure 2), with an observation window allowing for optical monitoring of the trapping sites.

Figure 1. A schematic image showing the design of the acoustic chip used for acoustic differential extraction. Miniature piezoelectric transducers are glued to a PCB providing fluidic and electrical connections. The surface of the PCB is cast in epoxy to create a flat surface used to dock an etched glass channel and thus create a sealed chip.

Figure 2. Sealed ADE microdevice in a brass interface (A). The device consists of a fluidic layer composed of borosilicate glass (B), and a printed circuit board layer containing three piezoceramic transducers (C).

The depth of the microfluidic channel is calculated to create an acoustic standing wave in the channel when activating the piezoelectric transducer. The standing wave creates three equidistant pressure nodes within the channel, half a wavelength apart; therefore the channel depth is 1.5λ. The working frequency of the piezoelectric transducers is 12.1MHz, and with an aqueous buffer the channel depth was therefore 183.5 µm. The channel width was 900 µm to make sure the transducers covered the entire channel width.

Sample is infused into the chip using pressure-driven flow from a syringe pump. When the ultrasound is activated at a specific frequency, objects passing the standing wave will be trapped in the pressure nodes in the channel above the transducer. The objects will levitate there with no contact with the channel walls until the ultrasound is switched off and the objects are transported away with the fluid flow for collection at one of the two possible outlets.

Polytetrafluoroethylene (PTFE) tubings (0.3 mm ID, 8.5 cm long) were attached to the male and female outlet on the chip for efficient transfer of product to a microcentrifuge tube for further processing off-chip.
**Acoustic differential extraction method**

ADE was performed in the sealed device as described above. The fluidic channels and tubing were evacuated of ultrasound gel using phosphate-buffered saline solution (PBS). Fluid flow was controlled using WPI syringe pumps model SP120-300 (World Precision Instruments, Sarasota, USA) in infusion and withdrawal mode at appropriate rates, as delineated below. The buffer inlets were connected to 1 mL Hamilton glass syringes (Hamilton company, Reno, USA). Sample was infused using a 100 µL Hamilton glass syringe. Microcentrifuge tubes were attached to the capillary tubing at each outlet for sample collection.

The ADE method consists of three steps: sample infusion/trapping, wash, and release (Figure 3). During the trapping and washing phases, the ultrasound was activated at 12.1 MHz (~6 Vpp) using a waveform generator (Agilent 33220A, Agilent Technologies, Inc., Palo Alto, USA). During the sample infusion/trapping of sample, focusing buffer was infused through inlet (B) at 6 µL/min, and sample was infused through inlet (S) at 1 µL/min; the sperm cells in the sample were collected for 6 minutes. The focusing buffer, a conventional differential extraction buffer25 devoid of the proteolytic digestion agent, was used to direct the infused sample to the center of the transducer and ensure a high trapping efficiency. Upon activation of the ultrasound, sperm cells were trapped while the unretained free DNA was directed toward the “female fraction” outlet. The fluidic control was performed using infusion of buffer through the side arm inlets (G1 and G2); a 1:5 (G2:G1) ratio of fluid infusion from the side arm inlets directed the lysate as described in the “Male and female fraction isolation through precise fluidic control” subsection (of Results) below. During the wash step, the ultrasound remains activated, and sperm cells remain levitated in the trap, while the focusing buffer (6 µL/min, 3.5 mins) perfuses the trapped cells. Upon completion of the washing, the ratio of flow rates from the sidearms is inverted (5:1, G2:G1), and the ultrasound is deactivated, resulting in release of the sperm cells and subsequent collection in the “male fraction” outlet (5 minutes). Sample withdrawal (0.5 µL/min) is implemented during the wash and release steps to prevent any sample, and thus, female DNA from entering the device and contaminating the male fraction.

![Figure 3](image)

**Figure 3.** The fluidic design of the ADE-chip. The sample in infused through inlet S and hydrodynamically focused by the buffer inlet B. The flow ratio between inlets G1 and G2 will decide if the sample will go to the male or female outlet.
Sperm cell trapping was viewed through an optical microscope (Leitz Orthoplan, Leica, Germany) and recorded to DVD via a CCD camera (Hitachi KP-D20BU, Tokyo, Japan) and DVD recorder (Panasonic DMR T2020, Sacaus, New Jersey).

Sample Preparation

Buccal epithelial cells and semen samples were obtained from healthy volunteers, as per IRB HIC approval #10896. Buccal epithelial cells (from a female) were obtained on cotton swabs and stored at room temperature until use. Semen was aliquoted and frozen until use. The cellular material was eluted from the swab in a conventional differential extraction buffer as developed by Gill et. al25, consisting of 10 mM trizma pH 8 (prepared via titration of trizma-base with trizma-hydrochloride), 10 mM EDTA, 0.1 M NaCl, 2% SDS, and 20 µg/mL proteinase K. Buccal swabs were incubated overnight at 56°C, during which the epithelial cells were released from the swab and lysed. A fixed volume of diluted semen was added to the lysed cellular material to simulate the male component of the sample.

Analysis of ADE product

Upon completion of the sperm cell trapping, conventional macroscale downstream analytical processes were utilized to demonstrate purity of the product. DNA extraction was completed using a QIAamp® DNA Mini Kit, (Qiagen, location) as per the manufacturer specifications.

PCR amplification was performed on a Perkin Elmer GeneAmp PCR System 2400 Thermocycler (Wellesley, MA). Short Tandem Repeat (STR) profile generation was completed using the COFiler® kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s specifications. The COFiler® kit contains primers to simultaneously amplify 6 STR loci: CSF1PO, D3S1358, TH01, D16S539, TPOX, and D7S820. In addition, a portion of the amelogenin gene is amplified. The 112 bp amelogenin Y chromosome fragment is male-specific. In addition, the STR profile, when compared to a reference sample, can be used to infer the source. Thermocycling consisted of 28 cycles of 60 s of denaturation at 94 °C, 60 s of annealing at 59 °C, and 60 s of extension at 72 °C. The amplification was begun with a 60 s, 95 °C initial denaturation and ended with 45 min at 60 °C. An ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) was utilized for separation of the PCR products under the following conditions: 5 s electrokinetic injection (15 kV), 25 min separation (15 kV), and Genetic Analyzer separation buffer (Applied Biosysytems). The capillary (50 µm ID, 36 cm effective length, 47 cm total length) was filled with POP-4 sieving polymer (Applied Biosytems) and maintained at 60 °C for the duration of the separation.

RESULTS AND DISCUSSION

The Acoustic Differential Extraction (ADE) technique

Herein, we demonstrate the viability of this alternative method of forensic differential extraction by showing: (i) selectivity of sperm cell trapping, (ii) efficient release of trapped product, (iii) product isolation through fluidic control mediated by laminar flow valving, and (iv) separation efficiency (i.e., purity of separated products).

Design and Fabrication Characteristics for Effective Trapping

The physical dimensions of the fluidic channel are of seminal importance in achieving a stable trapping system with good separation efficiency. To achieve the desired trapping zones in the channel, the channel must have a height that corresponds to a one and a half wavelength of the transducer frequency. Another important aspect is the thickness of the reflector, which should have a thickness corresponding to an odd multiple of a quarter wavelength. If these desired
dimensions are not achieved during the glass etching, the system will be improperly tuned and the trapping zones may appear closer to or at the glass or transducer surface. A trapping zone close to a surface may result in cells contacting the surface, thus, enhancing the possibility for adsorption and negatively affecting release upon deactivating of the ultrasound - supplementary video 1 shows this poor release of cells trapped in an unoptimized system. This can best be summarized as the actual trapping force will be weaker in an unoptimized system, which may negatively affect the separation efficiency.

**Modeling and simulation**

In order to optimize the fluidic control of the system, the laminar flow valving was modeled and simulated using COMSOL Multiphysics before implementing the design. The two-dimensional model used a transient Navier-Stokes module to describe the microfluidics and the Convection-Diffusion module to simulate the female cell lysate. A post-processing particle tracing was used to simulate the movement of the sperm cells upon release from the ultrasonic trap (Figure 4). A constant concentration, seen as a color gradient in figure 4, was infused through the sample inlet (S) and focused using the buffer inlet (B), thus simulating the female cell lysate entering the chip. The ratio between the buffer inlets G1 and G2 was modulated to direct the sample to either the female or male outlet. Different channel designs and flow ratios were tested before choosing the design later implemented. A flow ratio of 1:3 allowed for precise fluid control without any contamination in the model.

![Figure 4](image)

*Figure 4. The complete simulated geometry showing the inlets and outlet is seen in (a). The inserts show the velocity flow lines for the flow controlling buffer inlets G1 and G2. In (b) the particles are fixed, simulating the trapping phase, while the cell lysate is injected and directed to the right, female, outlet. Insert (c) shows the velocity field after switching the flow ration between G1 and G2, now directed the released particles to the left, male, outlet.*
Male and female fraction isolation through precise fluidic control

Precise fluidic control in any microdevice is essential to its success. In this particular application, the effectiveness of the trapping and release of cells is irrelevant if the recovery of the purified fractions cannot be achieved. It was clear that the utility of the ADE device for adaptation in the forensic sector would be defined by the simplicity of a fluidic control that allowed for efficient recovery of the male and female fractions following trapping. There are numerous means of fluidic control have been described in the literature including the use of active valves and pumping\textsuperscript{26-29} as well flow switching\textsuperscript{30}. We have shown that one form of active valving, using a PDMS elastomeric membrane as described by Grover et al\textsuperscript{27}, could be very effective for fluidic control in integrated multiprocess devices\textsuperscript{28, 31}. However, for the simplicity of the ADE microdevice, we chose to adapt an elegant method of ‘laminar flow valving’ originally described by Blankenstein et al\textsuperscript{30}. Laminar flow valving involves a simple form of valving that exploits laminar flow and does not necessitate physical valves that require actuation. This was thought to be ideal for the ADE as it circumvents the use of PDMS (or other substrates) to which biological material may adhere. With this fluidic control scheme, both the female and male fractions could be spatially resolved into different flow paths on the ADE chip. The design of the channel structure, as shown in Figure 4, allows the flow to be directed towards either of the two outlets using only the adjustable ratio of flow rates from the two buffer inlets (G1, G2).

The design simulated and shown in figure 4 was wet-etched in glass and docked with the acoustic platform in order to evaluate the performance. Syringe pumps were connected to the two sidearm buffer inlets (G1, G2), and through control of the flow rate ratio of G1:G2, the outlet was selected. As opposed to the suggested 3:1 or 1:3 flow ratio suggested in the simulation, a ratio of 5:1 or 1:5 were chosen. This flow rate ratio ensured effective recovery of the entire sample in either the male or female outlet. Supplemental video 1 shows the control with which the a dye solution can be directed to the desired outlet by altering only the flow rate ratio of G1:G2.

Once fluidic control was demonstrated, we utilized a solution to simulate a sexual assault sample for the purpose of illustrating the direction of fluid flow, sperm cell trapping and subsequent recovery of the highly-purified male and female fractions. The simulated sexual assault sample was created using 10 µm polystyrene microparticles to represent sperm cells and a colored dye (Evan's Blue) to represent the vaginal epithelial cell lysate (including free DNA from the female). Figure 5 shows the infusion of the simulated sexual assault sample and the use of laminar flow valving for fluidic control during the trapping and separation of the male and female fractions. Before starting the sample infusion, the ultrasound was activated at 12.1 MHz. Upon infusion of the simulated sample, the polystyrene particles were trapped over the transducer while the dye, unretained by the ultrasonic trap, was directed to the female outlet reservoir as shown in Figure 5a. The trapped particles were washed (Figure 5b) by infusing buffer through the buffer inlet (BI, as shown in Figure 1) to remove any inadvertently trapped free DNA from the female. The flow was redirected by inverting the ratio of buffer infusion through the two buffer inlets (G1 and G2, as shown in Figure 1) to the sperm cell outlet and the ultrasound deactivated in order to collect the particles (Figures 5c & 5d). As can be seen in figures 5c and 5d, a small amount of blue dye remains in the channel during the washing and release phase. This effect is cause by the focusing buffer dragging small amounts of sample from the sample inlet. By implementing a slow withdrawal of the sample during the washing and release, this effect could be minimized.
Figure 5. A series of still microphotographs of a simulated sexual assault sample, demonstrating trapping and fluidic control using laminar flow valving. 5(a) A mixture of Evan's Blue dye and microparticles is infused into the device. The particles are trapped above the transducer in the acoustic standing wave, and can be seen as a dark clusters over the squared transducer area, while the dye is washed into the right outlet. (b) After 1 min of sample infusion, the sample flow is switched off and the channels are washed with buffer. (c) When washing is complete, the outlet flow is redirected to the left outlet. The movement of the dark cluster between (b) and (c) indicates the release of the particles from the acoustic trap and in (d) the microparticles are directed to the left outlet.

Sperm cell trapping

Equation 1 describes the theory behind which cell/particles will be attracted retained or unretained in the acoustic trap; however, in practice, the characteristic particle trapping behavior needs to be determined empirically. Due to the different physical properties of sperm cells and epithelial cell lysate – mainly the difference in volume – it was hypothesized that sperm cells would be retained in the acoustic trap, while free DNA in the epithelial cell lysate would be unretained. Studies were performed using mock sexual assault samples consisting of a mixture of epithelial cell lysate and sperm cells. Upon activation of the ultrasound (12.1 MHz, 6 Vpp), sperm cells were trapped in the nodes of the standing wave above the microtransducer. After infusion of the sample, the trapped sperm cells were washed with buffer to ensure any inadvertently-retained DNA (from lysed female epithelial cells) was removed from the channel. All unretained material (including free female DNA) was collected in the ‘female outlet’. Following the washing step, the flow was directed using laminar flow valving and the ultrasound deactivated, allowing the released sperm cells to be directed to the ‘male outlet’ for collection of the enriched male fraction.

Sperm cell release

Efficient release of sperm cells from the acoustic trap is essential to the development of this device. Any loss of biological material equates to a loss of evidentiary material and, potentially, the failure to generate enough DNA for a perpetrator DNA profile. The ADE method resulted in efficient release of sperm cells from the acoustic trap, as judged through an optical microscope. (See supplemental video 2.) Sperm cells were cleanly released from the trap because the cells are trapped in three-dimensional fluidic space at the nodes of the standing acoustic wave as opposed to the surface of the glass. As a result, any issues that may be encountered with cell adsorption to the glass is avoided. This is in stark contrast to other cell trapping and separation methods such as dielectrophoresis, in which the cells are trapped at the surface of the device and, hence, tend to adsorb to the substrate, making release and subsequent recovery of the entire sample difficult32.

In the design of the ADE microdevice, we recognize the potential for free DNA to adsorb to the microchannel surface. A variety of surface coatings (such as polydiallyl dimethyl ammonium chloride or polybreen) could be utilized in such a manner, should the need arise. In
forensic samples, this is not a likely scenario as the source of free DNA is from the victim’s epithelial cells, whereas, typically, the limited DNA in the sample is from the male. In the ADE method, the sperm cells remain intact and are trapped in the center of the microchannel -- therefore, are not subject to such consideration.

**Purity of the separation products**

A mock sexual assault sample (lysed buccal epithelial cells with sperm cells added to the lysate) was utilized to demonstrate the utility of the ADE technique to forensic casework. This sample simulates the typical vaginal swab obtained from a sexual assault victim, but is not a post-coital sample due to the difficulty in obtaining such a sample. The total genomic DNA and total male DNA were quantitated for each sample (pre- and post-ADE) to determine the enrichment of male or female DNA in each product.

The ultimate assessment of the ADE device is the amplification and separation of a forensic DNA profile to demonstrate the purity of the recovered male and female fractions. By conventional means, a forensic short tandem repeat (STR) profile was produced off-chip by PCR amplification with a commercially-available kit used in forensic laboratories. The resulting STR profiles from this sample before and after ADE are shown in Figure 6. The STR profile resulting from the original mixed cell sample (Figure 6A) is approximately 26 ± 2.0% male, as determined by comparison of this profile with those of the male and female cell donors (data not shown), and calculated using the peak area ratios of the male and female alleles. Via ADE, the male and female fractions obtained are enriched for male and female DNA, respectively. The STR profile of the biological material collected from the ‘male outlet,’ as shown in Figure 6B, shares common alleles at all loci with that of the male donor (data not shown) indicating that they are from common origin (92 ± 7.9% male as determined by peak height ratios). In addition, the STR profile of the biological material collected from the ‘female outlet,’ as shown in Figure 6C, shares common alleles at all loci with that of the female donor (data not shown), indicating that they are from common origin (95 ± 4.1% female as determined by peak height ratios). The data shows that the ADE device provides effective separation of male sperm cells and female epithelial cell lysate, as a purified male identification profile was obtained where it was otherwise uninterpretable.
Figure 6B: STR profile of biological material collected from ‘male outlet’:

Figure 6C: STR profile of biological material collected from ‘female outlet’:

Figure 6. Forensic DNA analysis (STR profiles) of recovered male and female fractions before and following ADE. (A) The STR profile of mixed cell sample prior to ADE. Comparison of this profile with those of the male and female donors indicates that the original sample is 26 ± 2.0% male. (B) STR profiles of the biological material collected from the ‘male outlet.’ The profile shares common alleles at all loci with that of the male donor, indicating that they are from common origin (92 ± 7.9% male as determined by peak height ratios). (C) The STR profile of the biological material collected from the ‘female outlet.’ The profile shares common alleles at all loci with that of the female donor, indicating that they are from common origin (95 ± 4.1% female as determined by peak height ratios).
In comparison to other microscale and macroscale methods for cell sorting and cell selection, ADE provides the quintessential method, circumventing the major drawbacks associated with the conventional method, as well as those reported in literature or suggested by the forensic community. For example, ADE does not require fluorescent tagging of cells, nor does it necessitate the use of immunoreagents for sperm cell retention via antigenicity. Since the initial development of what has now become ‘conventional differential extraction’, a limited number of alternatives have been reported that specifically address the analysis of sexual assault evidence. In one report33, a filtering method has been demonstrated, although clogging of the filter and poor separation purity were problematic. An alternate method, laser-based microdissection of sperm cells, provided an interesting approach but not one that was applicable to high-throughput sample processing. Fluorescence-activated cell sorting (FACS) has also been applied to forensic differential extraction to sort the cells by ploidy34, although this method requires fluorescent-staining of the intact cells. The only microscale technique reported, separation by differential sedimentation, also requires intact epithelial cells, which is not always achievable depending upon degradation of the evidentiary material.

The ADE method is highly-versatile, in that any size of evidentiary sample (µL to mL) is permissible. The flow rates and channel architecture can both be optimized to increase the capacity for a large range of sample sizes. In addition, one could envision a device containing multiple transducers, if need be, to split the sample among several channels for enhancing the throughput and capacity of the device without significantly increasing the analysis time. The method has been developed with direct integration to downstream processing steps in mind. That is, the side-arm inlets (G1 and G2) were designed for addition of reagents for solid-phase extraction of DNA. Although integration with subsequent sample processing steps (like DNA purification) is not required for use in a forensic laboratory, the true potential of the method will be fully realized upon such integration.

The optimal design for the introduction of ADE into forensic laboratories will, likely, involve engineering improvements such as enhancing the disposability of the device. In a forensic laboratory, the device cannot be reused due to the possibility of sample contamination. Therefore, in order to decrease the cost of the device, we speculate that the printed circuit board layer be developed as part of the ‘docking station’, with the channel structure as a sealed layer, rather than the PCB layer be in fluidic contact with the sample. Further work in this direction is currently underway.

CONCLUSION

The ADE method described here has been shown to be applicable to forensic differential extraction of sexual assault evidence by demonstrating sperm cell trapping from a mixture, efficient release of the trapped cells, and precise control of the fluidics for isolation of the highly-enriched product fractions. The purity of the male and female fractions was demonstrated using real-time PCR as well as forensic STR profiling, the forensic “gold-standard” of purity.
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ACOUSTOPHORESIS IN WET-ETCHED GLASS CHIPS

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ABSTRACT

Acoustophoresis in microfluidic structures has primarily been reported in silicon microfabricated devices. This paper demonstrates, for the first time, acoustophoresis performed in isotropically etched glass chips providing a performance that matches the corresponding silicon microdevices. The resonance mode characteristics of the glass chip were equal to the silicon chip at its fundamental resonance. At higher order resonance modes the glass chip displays resonances at lower frequencies than the silicon chip. The cross-section profile of acoustically focused particle streams are also reported for the first time, displaying particles confined in a vertical band in the channel center both for glass and silicon chips. A particle extraction efficiency of 98 % at flow rates up to 200 μl/min (2 % particle concentration) is reported for the glass chip at the fundamental resonance. The glass and silicon chip displayed equal particle extraction performance when tested for increasing particle concentrations of 2-15%, at a flow velocity of 12.9 cm/s for the glass chip and 14.8 cm/s for the silicon chip.

INTRODUCTION

Continuous separation of cells and particles by means of microsystem technology and microfluidics is gaining increased interest in the biomedical and biochemical field. Several techniques are available that either use externally induced forces as the separation mechanism or the geometry of the microfluidic device itself as the separating element. Magnetophoresis1-3 and dielectrophoresis4-6 are using magnetic and electric fields respectively while pinched flow fractionation7-9, hydrodynamic filtration10-12 and obstacle induced separation13-15 relies on the combination of laminar flow and the channel geometry. Another alternative for separation with externally induced forces is the use of acoustic forces, acoustophoresis, which is the topic of this paper.
Acoustic forces in microsystems have proven to be a very versatile and gentle tool for on-chip handling of particles and cells. The forces created by standing waves can be used for such diverse tasks as eliminating lipid emboli from shed blood in thoracic surgery, blood component fractionation and particle sizing, contaminated blood plasma replacement, and buffer media exchange. Specific selection of affinity binding phages from bacteriophage libraries, rare event selection, positioning, and trapping have shown that no adverse effects related to the acoustic handling can be seen on cells.

Commonly, the standing wave is formed between two parallel walls, using one wall as an ultrasonic transmitter and the other wall as a reflector. Rectangular geometries with vertical walls have most commonly been used, but systems using focused sound waves have also been reported. The standing wave can be formed either between the bottom and the top of a fluidic channel, between the side walls or as a combination of both to create a two-dimensional force-field.

The way of actuation depends both on the chip design and in which direction the force-field should be active. The most straightforward approach is to position the ultrasonic transducer with its direction of transmission parallel to the desired force-field direction, figure 1A. It is however also possible to create a lateral resonance perpendicular to the direction of transmission, by positioning the transducer as seen in figure 1B. This can be done with a standard transducer, a shear transducer or using wedges to increase the amount of shear waves induced, see figure 1C.

Figure 1. A) The acoustic standing wave is induced parallel to the wave propagation. B) The standing wave is induced perpendicular to the primary wave propagation. C) A wedge is used to couple the waves into the cavity.

Most devices using lateral resonances are fabricated in silicon using either anisotropic wet-etching or deep reactive ion etching (DRIE). As silicon is a relatively expensive material and the fabrication process requires mask aligners for precise pattern transfer, alternative materials with easier fabrication processes are of interest. An alternative way of fabrication is by creating a sandwich-structure with different layers defining the dimensions of the channel as well as the chip. The precision in such a method is, however, limited and may cause problems when small tolerances are requested.

Polymers and glass are becoming preferred material bases in chemical and biomedical microdevices as they offer cheaper and less complex fabrication means. Designing polymer based microdevices for ultrasonic actuation is, however, a non trivial task due to the large acoustic attenuation. In this perspective, glass has several attractive properties beyond low cost such as optical transparency, hydrophilic surface properties, electrical insulation and good chemical resistance. Glass also bonds well to silicon and has a very low acoustic attenuation while being amenable to a multitude of micromachining processes. Isotropic wet-etching is the most widely used fabrication method offering high
performance at low cost. For more complex structures, DRIE\textsuperscript{37} and molding/hot embossing\textsuperscript{38} are also possible but both techniques are more laborious and require advanced process equipment. Powder blasting can be employed to manufacture simple structures such as through holes and channels with limited demands in surface smoothness\textsuperscript{39}.

Using glass as the bulk material enables the use of microscopy techniques that have not been possible to use in previously reported acoustic silicon devices. One example is phase contrast imaging, a method commonly used to image cells or other small, transparent objects. Another example is Raman spectroscopy, where recent development projects cancer cell detection in clinical samples\textsuperscript{40}. Raman spectroscopy can be anticipated to be combined with acoustic microchip cell handling for label-free on-chip cell sorting. The possibilities in achieving optimal light conditions are also vastly increased when a completely transparent material is used and both reflective and transmission microscopy can be used.

The technique of manipulating cells and particles in the microfluidic domain with acoustic forces relies on an acoustic standing wave generated in an enclosed fluid volume on a chip. The magnitude of the radiation force acting on the particles is strongly dependent on the radius of the particle (to the third power) and proportional to the acoustic frequency\textsuperscript{41, 42}. The relationship between the density and compressibility of the particle and the surrounding medium will determine if the particle will move toward a pressure node or an antinode in the standing wave. For instance, a particle suspension flowing through a microchannel with a width of a half wavelength will experience a radiation force concentrating all particles into a focused band in the center of the channel, see figure 2. The laminar flow in the microchannel prevents the particles from dispersing again and if the microchannel ends in a three way split, the concentrated particles can be collected via the central outlet while the clear medium exits through the side outlets and a simple separation/concentration step has been achieved.
Figure 2. A schematic of the channel design and cross-section of the different separation chips investigated. Figure A) is the anisotropic silicon chip, B) the isotropic 1MHz glass chip and C) is the actual shape of the cross section of the 2MHz glass chip.

Our most recent experimental data demonstrate that it is possible to design isotropically wet etched glass devices that has particle manipulation properties comparable to what previously only has been reported for silicon-based substrates. This paper presents for the first time piezoelectrically actuated particle separation in an isotropically etched all glass microfluidic chips with separation properties matching the performance of silicon based acoustic separators. The performance of the glass chip is compared to the corresponding anisotropically wet-etched silicon chip in a continuous flow separation application. A continuous medium exchange with live cells using acoustic forces in a glass-chip is also presented.
MATERIALS & METHODS

Design and fabrication

A basic chip design with three inlets, three outlets and a separation channel was used to compare the performance between the anisotropic silicon chip and the isotropic glass chip, see figure 3. The separation performance was measured by focusing and separating 5 µm polyamide microparticles from their suspending medium. The particle suspension was a commercially available blood phantom (Danish Phantom Design, Jyllinge, Denmark), designed to mimic the viscosity, density and sound velocity of human blood.

The glass and silicon chips were both designed to have a lateral resonance at 2 MHz. In order to achieve a single node at 2 MHz, the channel widths were designed to be 375 µm, i.e. λ/2 at 2 MHz in water. As the glass chips have an isotropic cross-section, the widest part of the channel was designed to be 375 µm. Both chips were etched to a depth of 125 µm and had a 30 mm long separation channel. The glass chip was provided with separate connections, figure 3B, to the side inlets and outlets, whereas the corresponding in- and outlets in the silicon chip were united to one single outlet, figure 3D.

The silicon separator was fabricated by means of double sided photolithography and anisotropic wet etching using KOH. The chip was sealed by anodic bonding of a glass lid. A more detailed description of the microfabrication of the silicon chip can be found.
in Nilsson et al. The cross-section of the anisotropic separation channel was rectangular with vertical walls, see figure 4A.

Figure 4. SEM images showing the cross-section of the silicon (A) and glass (B) channel.

The glass chip was fabricated by wet-etching 0.7 mm thick borosilicate chromium blanks (Telic Company, Valencia, USA), pre-coated with 0.5 µm positive resist, using a HF:HNO$_3$:H$_2$O mixture. After etching, fluidic access holes were drilled using a 1 mm diamond glass drill. The etched and drilled chip was then cleaned in KOH in an ultrasonic bath and was thermally bonded to a clean borosilicate glass lid to form a sealed chip. Silicone tubing was glued to the fluidic access holes to hold standard 1/16” Teflon tubing.

When etching glass in hydrofluoric acid, the resulting shape of the side-walls depends on different parameters. The adhesion between the masking layer and the glass, the pH or temperature in the etch solution and the amount and type of stirring used are examples of parameters that can change the shape of the side-walls.

The etched 2 MHz glass channel did not have the typical isotropic side-wall geometry, see figure 4B. This is most likely caused by limited convective exchange of etch components in the zones under-etching the mask. A similar channel with a twice as wide mask, with all other parameters unchanged, resulted in a perfect semi-circular side-wall.
**Instrumentation**

The chips were actuated using an 11 x 32 mm external PZT piezoelectric transducer (PZ26, Ferroperm Piezoceramics, Kvistgard, Denmark) with a thickness of 1 mm, corresponding to a fundamental resonance of 2 MHz. The transducer was applied to the backside of the chips using ultrasonic gel for good acoustic coupling and clamped in place, see figure 3A. The transducer was actuated using a Hewlett Packard 3325B waveform generator and an Amplifier Research 75A250 amplifier. The input power to the transducer was set at 0.5 W for all experiments and monitored using a Bird model 5000-EX digital power meter.

The confocal image data was acquired with an Olympus BX51WI microscope using the Fluoview 300 software. Fluorescent particles with a diameter of 4.1 µm were focused using the acoustic standing waves and images of the focused particle band were taken at different depth in the channel using a motorized microscope-stage and a step-size of 10 µm. For each confocal image, the line-scans were averaged to obtain the intensity profile of the lateral particle distribution and the full-width half-maximum (FWHM) and maximum intensity were calculated, see figure 5. Using these data, a corresponding image was created in Adobe Illustrator where the FWHM-value was used for the width of the particle band and color intensity of the band was correlated to the maximum intensity value for each confocal depth.

![Image](image.png)

**Figure 5.** The line-scans in the fluorescent image, top image, are averaged to achieve an intensity plot of the lateral particle distribution, lower image. For each confocal depth, the corresponding average full-width half-maximum value (FWHM) is calculated. The FWHM-value is used as a representative width of the particle band, intensity-coded using the maximum intensity.
The separation efficiency was measured using a Beckman Coulter Multisizer 3 where the amount of particles in the center outlet was compared to the total amount of particles in all outlets. The samples were collected using 50 µl loops attached to an automated switch valve (Valco Instrument Company Inc.) while running the continuous separation.

Blood washing and medium exchange

A continuous medium exchange with human blood was performed as an alternative mode of performance evaluation. Evans blue (Merck AG, Darmstadt, Germany) was used to simulate a contaminant and was added to human whole blood. The erythrocytes were diluted to a hematocrit of 2 % using pure plasma and 180 µg/ml of Evans blue was added. The contaminated blood was infused through the side-inlets at 70 µl/min while clean plasma was infused through the center inlet at 130 µl/min. The acoustic forces moved the erythrocytes from the contaminated plasma into the clean plasma fraction at the center of the channel, figure 6. An input power of 0.8 W (8 Vpp) was used to ensure a good focusing of cells in the channel center. The erythrocytes transferred to the clean plasma were collected via the center outlet at 70 µl/min while the contaminated plasma continued to the side-outlets and was withdrawn with 65 µl/min in each outlet. The different infusion/withdrawal rates ensured that a minor part of the clean buffer zone was directed to the side outlets and reduced the risk of contaminants spilling into the clean center outlet.
Figure 6. The blood wash and medium exchange principle. Contaminated blood enters from the side inlets and clean plasma enters through the central inlet. The blood cells are switched over to the clean plasma by the acoustic radiation force and exit through the center outlet while the contaminated medium exits through the side outlets.

The blood washing efficiency was evaluated using a Labsystems Multiskan Multisoft photometric plate reader at an absorption wavelength of 595 nm. The ratio of absorbance in the contaminated media and the washed blood was compared and an average of six different samples was calculated.

RESULTS AND DISCUSSIONS

Resonance mode characterization

By visual inspection, the best particle focusing for the chips occurred at 2.063 MHz for the silicon chip and at 2.214 MHz for the glass chip. The particles were focused into the center of the channel with what appeared to be a uniform distribution of particles along the depth of the channel, much like the schematic image shown in figure 2. To verify the particle distribution, a confocal microscopy scan was performed on fluorescent particles focused at two different flow velocities for each device. The result, seen in figure 7, shows a slight bulging of the particle band in the center of the channel for both chips. This is most likely explained by the parabolic flow-profile, giving a maximal flow velocity in the center of the channel causing particles there to be exposed to and focused by the acoustic standing wave for a shorter time period. The color intensity in the figure is a measure of the particle density, thus being higher in the tightly focused areas as compared to the widened zones.

Figure 7. A cross-section view of the band-formation in the channels based on confocal image data. The width of the particle band is decided by the FWHM-value and the intensity is correlated to the maximum intensity from the averages line-scan. The widening of the particle band in the center of the channel is caused by the parabolic flow profile causing the particle in the center to have less time to focus into a narrow band.

Using COMSOL Multiphysics, an eigen-frequency analysis of the two chips was performed. The simulations predicted a fundamental frequency of 2.36 MHz for the glass chip and 2.0 MHz for the silicon chip. The squared pressure amplitudes, proportional to
the acoustic radiation force, show a single pressure node in the center of both channels, see figure 8. The simulations predicted the harmonics to appear at 4, 6 and 8 MHz for the silicon chip while the glass chip showed a different behavior with harmonics at 4.3, 5.7 and 7.4 MHz.

Figure 8. The squared pressure field, proportional to the acoustic force, in the glass cavity (A) and the silicon cavity (B) as simulated in COMSOL Multiphysics using eigen-frequency analysis. The simulation predicts the fundamental frequency to occur at 2.36 MHz for the glass chip and 2.0 MHz for the silicon chip.

The glass and silicon chip showed similar behavior in focusing particles when operated at their fundamental frequency, see figure 9A and 9E. The particle suspension used was well focused and exited through the center outlet as expected. The first harmonic, twice the fundamental frequency, was tested to focus the particles into two bands, figure 9B and 9F. The glass chip displayed a slightly lower focusing efficiency, with broader particle bands, at this frequency but the difference between the two materials was marginal.
Figure 9. Particle focusing at higher harmonics; the glass chip at the top (A-D) and the silicon chip at the bottom (E-H). The channel walls have been outlined to improve the visibility.

At the second harmonic a more distinct difference between the chips was seen. The glass chip deviated from the expected frequency of 6.6 MHz and displayed a resonance at 5.515 MHz instead which correlates well with the resonance frequency predicted by the simulations. The focusing was however superior to the silicon chip where the flow rate had to be lowered to 0.02 ml/min at 0.5W acoustic input power in order to get a reasonable picture of the band formation, which should be compared to 0.18 ml/min for the glass chip, figure 9C and 9G.

The same trend was repeated at the third harmonic where it hardly was possible to achieve a four band focusing for the silicon chip while the glass chip clearly showed a better focusing, figure 9D and 9H. The resonance frequency for the glass chip again differed from the expected 8.8 MHz and was now found at 6.702 MHz.
The reason for the deviation in resonance frequency is not clear but is most likely associated with the tapered shape of the side-walls. For a rectangular geometry there is an analytical solution to the different lateral resonances. An isotropic channel is however more complex and the resonances can only be determined by simulations. As a comparison, a wet-etched glass chip with a fundamental resonance at 1.0 MHz, thus with twice the width of the 2 MHz chip, was tested. This chip showed a very nice isotropic cross-section as opposed to the 2 MHz chip, see figure 2B. The 1 MHz chip performed a single band focusing at 1 MHz and was as expected slightly inferior to the 2 MHz chip in focusing strength due to the lower frequency. However, all of the harmonics for this chip was at the expected multiples of the fundamental frequency: 2.0, 3.0 and 4.0 MHz respectively. Clearly, more work needs to be done in this area to fully understand how the geometry of the cross-section of the chip affects the resonance frequencies.

**Particle focusing efficiency**

The separation efficiency of the particle separation in both glass and silicon chips versus an increasing flow velocity can be seen in figure 10. The separation efficiency clearly decreased as the flow velocity increased since the particles spent shorter time in the acoustic force-field. At flow velocities up to 10 cm/s, the separation efficiency of the glass and silicon chip was comparable, however at higher flow velocities the performance of the glass chip decreased as compared to the silicon chip.

![Figure 10](image)

**Figure 10.** The separation efficiency versus the flow velocity at a particle concentration of 2% and an acoustic power input of 0.5W. The performance of the two chips is similar although the silicon chip has slightly higher separation efficiency at higher flow
velocities. The flow velocities used correspond to volumetric flow rates of 50 – 700 µl/min.

While performing the experiments, volumetric flow rates were set on the syringe pumps. Due to the different cross-sections of the chips, the flow velocities and thus the retention times of the particles will be different in the channels. The corresponding flow velocities were therefore calculated using a three-dimensional COMSOL-simulation. For a volumetric flow of 100 µl/min, the glass-chip has an average flow velocity of 6.45 cm/s, while the silicon-chip, with a larger cross-section, has an average flow velocity of 3.7 cm/s. Thus, for a given volumetric flow rate, the silicon chip will have a slower velocity and particles will spend more time in the force field.

The volumetric flow rate is many times a crucial factor and commonly in microfluidic applications a volumetric flow of a few hundred microliters per minute is more than sufficient. If even higher flow rates are needed, several parallel channels actuated by a single transducer can be used\textsuperscript{17}. It is also possible to increase the input power and create a stronger force field that will work even for higher flow rates. A higher input power may however, result in an elevated temperature in the channel due to thermal losses in the transducer. This effect can be counteracted by using heat-sinks or Peltier-elements and work as a method of controlling the thermal environment on-chip\textsuperscript{26, 28}.

The separation efficiency for the silicon and glass chip with regards to the inlet sample concentration was compared, see figure 11. As expected from earlier tests with silicon separation chips\textsuperscript{35}, the separation efficiency decreases with particle concentrations ranging from 2-15 %. The silicon and the glass chip show almost the same behavior for increasing concentration.
Medium exchange in cell washing

Acoustic manipulation of cells offers a mode of non-invasive and non-contact spatial localization of cells in microfluidic systems. Most importantly, it has been shown in several experiments that acoustic cell manipulation does not induce any traceable adverse reactions to the exposure to an acoustic force field 17, 26, 31. This fact opens up for the use of acoustic force manipulation to perform a set of unit operations that can be combined to design relevant bioanalytical sequences, integrated in a single microfluidic device. One such application is the possibility to perform a buffer exchange without having to expose the cells to a mechanically stressing event such as centrifugation. In this case a separation channel operated at its fundamental resonance and supplied with two buffer inlets as outlined in figure 6 can switch cells from one buffer medium to another by the acoustophoretic process investigated herein.

During the medium exchange, a separation efficiency of $92.8 \pm 3.9\%$ was achieved and $97.1 \pm 1.1\%$ of the contaminant was removed from the blood using the glass chip, at a blood sample input flow rate of 70 µl/min. This can be compared to a separation efficiency of $89.3 \pm 5.8\%$ and a contaminant removal of $92.3 \pm 1.2\%$ for the silicon chip, see table 1. If higher levels of medium exchange is requested it is advised that this process is repeated in a sequential step.

Table 1. A comparison in contaminant removal efficiency between a glass chip and a silicon chip.

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<tr>
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<th>Glass</th>
<th>Silicon</th>
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<tbody>
<tr>
<td>Separation efficiency</td>
<td>$92.8 \pm 3.9%$</td>
<td>$89.3 \pm 5.8%$</td>
</tr>
<tr>
<td>Contaminant removal</td>
<td>$97.1 \pm 1.1%$</td>
<td>$92.3 \pm 1.2%$</td>
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180 µg/ml of Evan’s blue was used as a contaminant and the blood was diluted with plasma to a hematocrit of 2%.

CONCLUSIONS

This paper reports for the first time an all glass microfabricated chip for acoustic force control of cells and particles in a continuous microfluidic process. Essentially equal performance of the glass chip and its silicon counterpart is reported. The possibility to use acoustic forces in wet-etched glass channels may make microchip-based acoustic cell and particle manipulation widely available to the bioanalytical microfluidics community. The micro-fabrication of glass is less complicated than the silicon fabrication and also requires fewer and less expensive instruments. Glass is also a cheaper material and the isotropic nature of the fabrication process allows for a larger degree of freedom in chip design.

The separation efficiency of the glass chips was above 97% for a 2% suspension for flow rates up to 200 µl/min. There are several ways of increasing the throughput in a
glass chip while maintaining the high separation efficiency. A rather straightforward approach would be to use several parallel channels but it is also possible to increase the input power. By increasing the input power the risk of elevating the temperature on chip is increased and measures to keep the temperature stable may have to be taken.

The acoustic glass separator has proven to be capable of handling the same tasks as the acoustic silicon separator, including particle separation and continuous medium exchange in human whole blood.

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SUPPORTING INFORMATION AVAILABLE

A video showing the focusing of a particle suspension in a 2 MHz glass chip. This material is available free of charge via the Internet at http://pubs.acs.org.

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