Acoustofluidics for flow cytometry and life science applications

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Acoustofluidics for flow cytometry and life science applications

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Abstract:

Acoustofluidics is a technology that utilizes acoustic waves in microfluidics for manipulation of cells and particles. The method has been proven effective for different unit operations in biomedicine, such as cell separation, enrichment and filtering. In this thesis, several applications of acoustophoresis are described, with technology development directed towards flow cytometry related applications.

The first paper describes a fluorescence activated cell sorter actuated by acoustic waves, capable of sorting micro particles by their fluorescence intensity, at a throughput of ~200 particles/s. The presented device utilizes continuous 2-dimensional acoustic focusing for particle alignment, and subsequentially sorts particles by short acoustic bursts upon fluorescence detection.

The analysis of non-spherical cells is known to be a challenge in flow cytometry, as the scattered light depends on a cell's relative orientation to the incident light beam. In paper 2, and novel method for controlled orientation of non-spherical cells (RBCs) is described. Using 2-dimensional acoustic standing waves, RBCs could be controlled to project either an edge-on, or face-on orientation towards a camera, during continuous flow.

In paper 3, a method for increasing the concentration of a cell sample is described. By combining a well proven acoustofluidic chip design, with a novel re-circulating fluidic architecture, the concentration of a cell sample could be increased with a factor of thousand or more, at flows up to 1ml/min in a single microfluidic channel.

Finally, paper 4 describes a method for measuring the hematocrit level (HTC) of a blood sample. The described device uses 1-dimensional acoustic standing waves to close-pack blood cells in a microfluidic channel. By imaging the microfluidic channel with a camera, the HTC level of a blood sample could be accurately determined in less than 60 seconds.

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Acoustic streaming around an air bubble

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List of publications

Paper 1. **Acoustic actuated fluorescence activated sorting of microparticles.**

Ola Jakobsson, Carl Grenvall, Maria Nordin, Mikael Evander and Thomas Laurell

Lab on a Chip, 2014. **14**(11): p. 1943-1950.

DOI: 10.1039/C3LC51408K

Paper 2. **Continuous Flow Two Dimensional Acoustic Orientation of Nonspherical Cells**

Ola Jakobsson, Maria Nordin and Thomas Laurell

Analytical Chemistry, 2014. **86**(12): p. 6111-6114.

DOI: 10.1021/ac5012602

Paper 3. **Thousand-Fold Volumetric Concentration of Live Cells with a Recirculating Acoustofluidic Device.**

Ola Jakobsson, Seung Soo Oh, Maria Antfolk, Michael Eisenstein, Thomas Laurell and H. Tom Soh

Analytical chemistry, 2015. **87**(16): p. 8497-8502.

DOI: 10.1021/acs.analchem.5b01944

Paper 4. **Rapid hematocrit level measurement in an acoustofluidic device**

Ola Jakobsson, Klara Peterson, Per Augustsson, Pelle Ohlsson, Johan Malm, Stefan Scheding, and Thomas Laurell

Submitted manuscript, Analytical Chemistry

The author's contribution to the papers included:

Paper 1. I fabricated the device, designed and assembled the experimental setup, performed the experiments and data analysis, and wrote the manuscript.

Paper 2. I fabricated parts of the device, designed and assembled the experimental setup, participated in the experiments, participated in the data analysis, and wrote part of the manuscript

Paper 3. I fabricated the device, designed and assembled the experimental setup, performed the experiments (except the viability study), participated in the data analysis, and wrote a major part of the manuscript

Paper 4. I fabricated parts of the device, designed and assembled the experimental setup, participated in the experiments, performed the data analysis, and wrote part of the manuscript.

Preface

This doctoral thesis is the first I have written in my life, and will presumably also be the last. I have tried my best to create a storyline relevant to the papers included. During my time as a PhD student, I have been given extensive freedom to explore many different areas in the research field of acoustofluidics. I have greatly appreciated this freedom, and found a great deal of interesting research topics. Maintaining cohesion has been one of the biggest challenges for me. The intended storyline throughout this thesis is the use of acoustofluidics, with a special interest in cytometry related applications.

Acoustofluidics is, in my opinion, an easy to use (if used correctly), robust and versatile tool for particle and cell manipulation in microfluidics. However, a tool is only useful if it can be applied to solve a problem. Acoustofluidics is in some sense a hammer looking for a nail. During my time at the department, I have devoted a lot of effort on learning how to use this hammer and improve it. More importantly, countless hours have also been spent on testing the hammer on different nails (read problems/applications). Some of the nails bent, but a few actually went in. I have also had the benefit of doing part-time work for Acousort AB, a start-up company developing Acoustofluidic technology for commercial use. To see some of my work being put to use outside the academic setting has been a great motivation for me.

To sum up five years, I have given a lot of presentations, written a ton of pages, spoken to a lot of people, spent a lot of time thinking, and most importantly performed hundreds of experiments. Although an unsuccessful experiment or even research project is not something to strive for, I believe it is something intrinsically linked with science. I have probably learned more from the failed experiments than the successful ones. Fortunately, success has not been completely absent. Some of the more successful experiments are included in this thesis, in the form of peer reviewed journal papers, and the less successful will be left untold (but hopefully not forgotten). In the end I know more now than when I started, and I sincerely hope that some of my scientific findings will benefit other people.

Contents

List of Abbreviations

ACF	Acoustic Contrast Factor
BAW	Bulk Acoustic Waves
BF	Brightfield (microscopy technique)
CCD	Charge Coupled Device
CTC	Circulating Tumor Cell
CV	Coefficient of Variation (relative standard deviation)
DEP	Di-ElectroPhoresis
DI-water	De-Ionized water. Also sometimes referred to as MQ (milli-Q) water
DLD	Deterministic Lateral Displacement (array)
FACS	Fluorescence Activated Cell Sorting
LOC	Lab On a Chip
MACS	Magnetic Activated Cell Sorting
PBS	Phosphate Buffered Saline (A typical cell medium)
POC	Point Of Care
PRF	Primary acoustic Radiation Force
PS	PolyStyrene
PZT	Lead (P), Zirconate (Z), Titanate (T). Sometimes used for Piezo ceramic transducer
RBC	Red Blood Cell
RT	Real Time
SAW	Surface Acoustic Wave(s)

1 Microfluidics

Introduction

Microfluidics can be defined as a technology that deals with the handling and manipulation of fluids at very low volumes. The devices or structures used for the handling of the fluids in microfluidics, typically have dimension and features within the micrometer range, and the volumes involved can range from atto (10^{-18}) [1] to microliters (μL). The most striking, and perhaps also most fascinating feature of microfluidics, is that some physical phenomena and forces that are normally not observed in the "macroscale world" suddenly becomes dominant.

An example of a phenomenon commonly being used in microfluidics is the filling of a micro capillary by capillary force. Water seems to flow upwards against gravity, when the small end of a hydrophilic capillary is brought into contact with water. If the capillary would be replaced by a larger tube, for example a drinking straw of similar material, no such effect can be observed. Another example which can be explained by the attributes of being in the microscale is the ability of *Aquarius remigis*, commonly known as the water strider, to be able to walk on water (Fig. 1). This ability can be explained by the surface tension of water, in combination with the long (nanostructured) hydrophobic legs of the insect. As the surface to volume ratio of an object increase as the size of that object is reduced, surface tensions forces are suddenly larger compared to gravitational forces, and an ability which sometimes in history have been explained by divine intervention and unnatural power, becomes natural and understandable with physics. Similarly to the water strider's ability to walk on water, microscale effects are utilized in the science of microfluidics in order to enable applications that are seemingly impossible when using macroscale "tools".

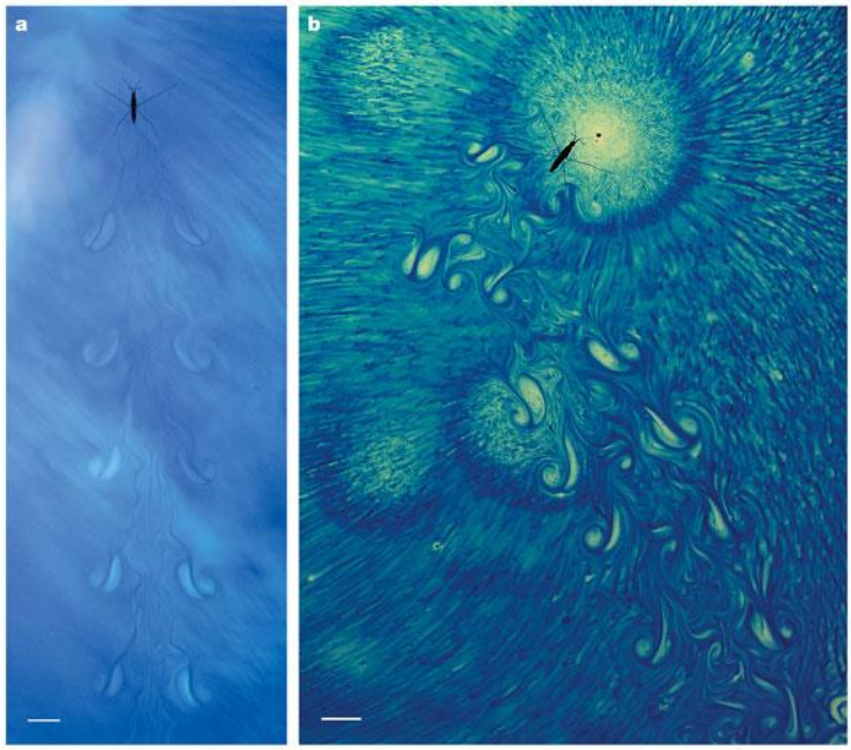


Figure 1. An image of water strider walking on water. The water is dyed with a thin top layer of thymol blue, which reveals the vortical footprints as the water striders propels itself forward. Reprinted by permission from Macmillan Publishers Ltd: Nature, Hu, D.L., B. Chan, and J.W.M. Bush, The hydrodynamics of water strider locomotion, reference [2]., copyright (2003).

The Lab on a Chip (LOC) concept (Fig. 2) is an extension of microfluidic technology. As microfluidics enables miniaturization of procedures in chemical or biological processes, several unit operations, can be fitted on a single microfluidic chip or cartridge, operating in a sequential or parallel manner. Typical unit operations are reagent mixing, incubation and separation processes. LOC devices normally facilitate automation and high throughput screening processes in chemistry and biological sciences.

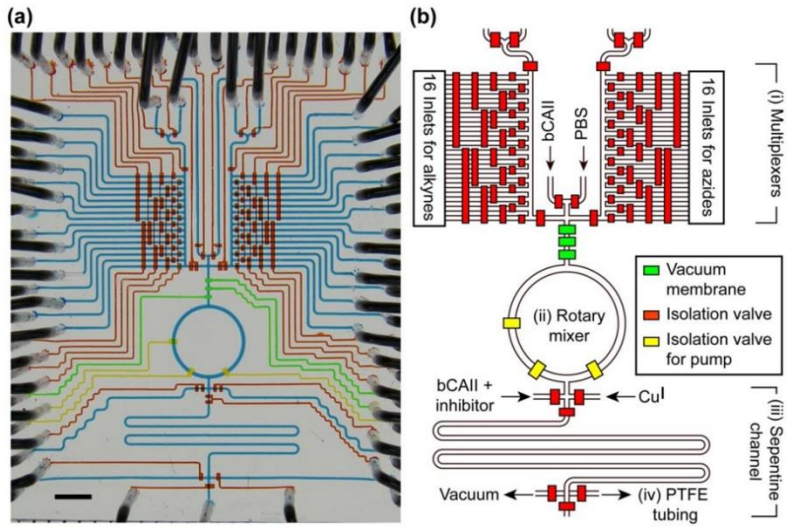


Figure 2. A microfluidic chip for chemical reactions, capable of carrying out 1024 parallel chemical reactions. Reprinted from reference [3]with permission from Elsevier.

Although microfluidics offer a toolbox with a great deal of advantages, there are naturally also challenges in using the technique. A very common challenge when using microfluidics is when a specific application requires the processing or handling of a large fluid volume. As a consequence, volumetric throughput (volume/time) is often a key parameter when the performance and feasibility of a microfluidic device is evaluated.

Some of the applications in our everyday life that are based on microfluidic technology are inkjet printers, and allergy and pregnancy tests. In medicine, mass spectrometry, flow cytometry and different types of blood analyzers etc. have improved the level of modern healthcare substantially. The field of microfluidics and all of its applications is too extensive to be covered in here, but an excellent paper capturing the highlights of the technology have been written by G. Whitesides [1].

Cell sorting and separation in microfluidics

A large faction of the microfluidics community is working with cell sorting and cell separation technologies. A commonly targeted and very important application, is the isolation of circulating tumor cells (CTCs) from blood for cancer diagnostics. Although cell sorting and cell separation essentially yields the same result, they are two very different methods. The following distinction between cell sorting and cell separation is made in this thesis:

Cell Sorting: In this thesis, cell sorting refers to a technique where each cell or particle is analyzed individually, and the collected information is used to make a logic sort decision in real-time (RT) such as yes/no (or maybe), followed by a sort event based on the logic decision. In biomedicine, cell sorting is typically performed with a fluorescence activated cell sorter (FACS) instrument. A macro-world analogy to cell sorting is when a person sort their socks after a laundry, for example by color. Each sock is visually inspected and manually (or automatically [4]) sorted into different drawers according to the obtained visual signal from the socks. An extensive review on microfluidic cell sorting techniques is available in chapter 4 of this thesis.

Cell separation: Cell separation essentially yields the same result as cell sorting, without the need of interrogating each cell on an individual basis. Cells can be separated based on their intrinsic or extrinsic properties, and their interaction with a specific device/object/field. Cell separation methods logically have the potential to have a much higher throughput compared with cell sorting methods, as it allows the analysis and sort logic process to be skipped. An analogy to cell separation is when yeast cells and other residues are removed from beverages by using a filter (a size based separation). Using a cell sorter for this purpose would result in a very expensive beverage. Among the microfluidic techniques that can be used to perform cell separations are acoustophoresis [5], magnetophoresis [6], deterministic lateral displacement arrays (DLD) [7], inertial microfluidics [8], dielectrophoresis (DEP)[9] and pinched flow fractionation [10] etc.. Other common separation processes used in biomedical labs, which are not based on microfluidics, are centrifugation and (despite the name) magnetic-activated cell sorting (MACS).

Laminar flow and Stoke's drag

Several effects occur in microfluidic systems, of which laminar flow and stokes drag are of particular relevance to this thesis. For a more extensive review of the physics at the microscale regime, see Squires et al. [11].

1.1.1 Laminar flow

Laminar flow is a phenomenon that can be observed in microfluidic structures. Laminar flow implies that a fluid flows in parallel layers, in a predictable fashion without observable irregularities or convective mixing between the layers. Turbulence in a flow can often be explained by inertial effects, which couples to the mass and velocity of an object or fluid, see fig. 3. Friction between parallel flow layers, (shear force) counteracts turbulence and facilitates predictable flow patterns. The shear forces acting on a fluid are linked to the viscosity of the fluid and wetted area of the microfluidic channel.

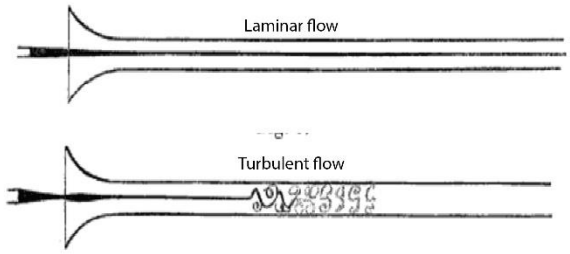


Figure 3. Laminar and turbulent flow as observed and depicted by Osborne Reynolds. Figure adapted from reference [12].

A method of predicting whether a flow is laminar or turbulent, is to calculate the Reynolds number of a flow system. The Reynolds number (Re) is defined as:

$$Re = \frac{\rho * \upsilon * D_H}{\mu}$$

Where ρ is the density of a fluid, υ is the mean velocity of the fluid, D_H is the hydraulic diameter of the system, and μ is the dynamic viscosity of the fluid. If Re<2000, laminar flow typically occurs. Fig 4. shows the laminar flow profile from a cross-sectional perspective in a microfluidic channel with typical dimensions of the channels used within this thesis.

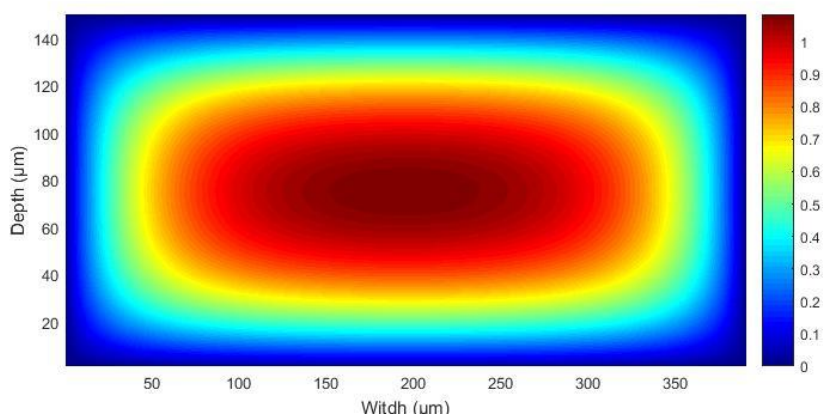


Figure 4. A color plot of the laminar flow profile in a microfluidic channel with a rectangular cross-section. The velocity of the fluid is indicated by color, where red represents a high velocity and blue low a low velocity.

1.1.2 Stoke's drag

Any particle moving through a liquid (being dragged), will experience a drag force in an opposite direction to the movement. The drag force F_{drag} (caused by "fluidic resistance") on a sphere is described by Stoke's law according to:

$$F_{drag} = 3\pi\mu Du$$

where D is the diameter of the particle and u is the relative velocity between the fluid and the particle. Stoke's drag typically becomes more dominant compared to other forces, when the size of an object is reduced. Stoke's drag is proportional to the diameter of an object (until a certain minimum), and most other forces (such as gravity) are proportional to the volume of an object.

2 Acoustofluidics

Introduction

Sound is essentially a mechanical vibration in a material, such as air or water. The human ear is capable of detecting and converting sound waves into neuronal signals in the range of approximately 20Hz to 20 kHz. Frequencies above and below the human audible range is defined as ultrasound and infrasound, respectively.

Since sound is a physical vibration (with momentum and inertia) in a material, it can be used to manipulate matter. Acoustofluidics is a specialized field within the microfluidics community, where sound waves are used to achieve spatial manipulation of suspended cells and particles within microfluidic structures. The word acoustophoresis, meaning "migration by sound", is a commonly used term by researchers in the field of acoustofluidics.

Theory

Sound waves propagating in a material can be characterized by their pressure and displacement component functions, typically sinusoidal in shape and phase shifted 90^{o} with respect to each other. Depending on the material properties of the medium the sound wave propagates in, the sound will propagate with a certain speed. The typical parameters used to specify the acoustic properties of a material is the bulk modulus and density.

When two or more sound waves meet, interference will occur, and the total pressure and displacement will be the sum of the contributing sound waves. If the sound waves interfering with each other has the same frequency, a standing wave will form. If a sound wave transitions through two materials with different acoustic impedance, part of the wave will be reflected according to the laws of reflection. An example of this is when a sound wave propagating in water meets a material with different acoustic properties, such as silicon or glass. In this case, the majority of the propagating wave will be reflected and the wave will interfere with itself, forming a standing wave. The theory for sound waves is well described in several physics textbooks [13, 14].

2.1.1 Resonance

Resonance is a phenomenon that occurs when energy is periodically applied to a system that has the ability to oscillate, store and transfer energy between different energy forms. Due to the ability to store energy, a resonant system oscillates with a high amplitude when actuated at specific frequencies. A classic example of a resonant system is the pendulum, which is able to store and transfer energy between kinetic energy and potential energy. When an external force is applied periodically

to the pendulum with the same frequency as the pendulum's eigenfrequency (natural frequency), the amplitude of the pendulum will increase. The eigenfrequency of a system is the frequency which that system tends to oscillate at without any externally applied forces.

There are many types of resonators, such as mechanical, electrical or optical. Many resonators can be modelled using the simple harmonic oscillator model, with a sinusoidal driving force. The gain (amplitude of system/amplitude of driving force) for a mechanic driven harmonic oscillator can be described by:

$$Gain = \frac{\chi}{\chi_0} = \frac{1}{\sqrt{(1-\tilde{\omega}^2)^2 + (\delta^2\tilde{\omega}^2)}}$$

Where $\tilde{\omega} = \omega/\omega_0$, and ω is the actuation frequency, ω_0 is the eigenfrequency of the system, χ_0 is the driving force amplitude, χ is the system amplitude, and δ is the damping coefficient/mass.

Naturally, all mechanical resonators are attenuated. Depending on the damping coefficient, the frequency response of the resonator has different characteristics. For a driven resonator at steady state, the input energy must equal the dissipated energy (per cycle). The Q factor of a resonant system can be defined as

$$Q = \frac{Energy\ stored}{Energy\ dissipated\ per\ cycle}$$

The Q factor is inherently dependent on the damping coefficient. In fig. 5, the gain for a simple harmonic oscillator is shown for different damping coefficients. For a low damping coefficient (high Q factor), the amplitude of the oscillator becomes very large when actuated close to its resonance frequency.

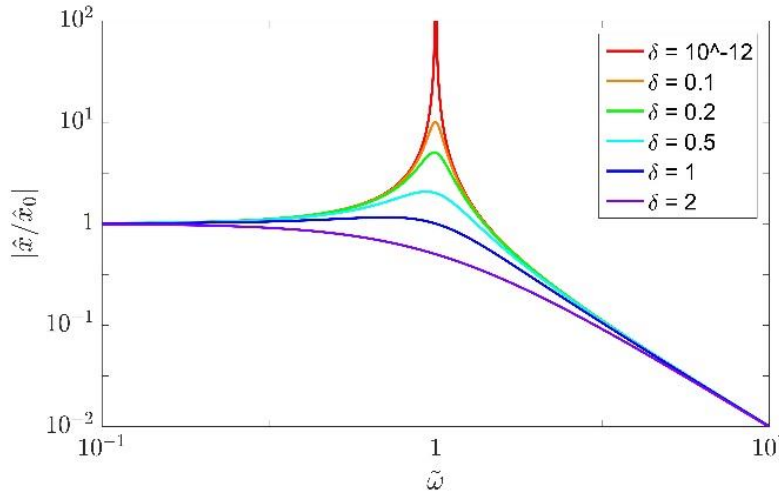


Figure 5. The gain of a driven simple harmonic oscillator for different dampening coefficients.

A resonating system can be characterized by its bandwidth, measured as the full width at half maximum (FWHM). Essentially, the FWHM describes the frequency range a resonator can be actuated at while maintaining half of the maximum amplitude. By definition, a more damped system has a higher bandwidth as compared with a less damped system, and is less (amplitude) sensitive to changes in the actuation frequency (or eigenfrequency). Parameters that might change the eigenfrequency of an acoustofluidic resonator are for example changes in temperature (of the bulk and channel medium), and speed of sound in the fluidic sample.

It should be noted that the simple driven harmonic oscillator model cannot be used to realistically describe the behaviour of the acoustofluidics systems used within this thesis. Nevertheless, it is reasonable to argue that if a high pressure amplitude is desired, a system with low damping should be used.

2.1.2 Primary acoustic radiation force

A solid particle suspended in a fluid (with different acoustic properties than the particle), interacts with sound waves propagating through the medium. In a standing wave, a net force on a particle can be generated for spatial manipulation. The primary acoustic radiation force and arises from the scattering of sound waves on the object suspended in the fluid. The theory and models describing the acoustic radiation force on a spherical particle in a standing wave field has been studied since the beginning of the 20th century [15-17] .

For a particle exposed to a standing wave, the primary acoustic radiation force F_{rad} can be described by

$$F_{\text{rad}} = 4\pi a^3 \Phi k_y E_{\text{ac}} \sin(2k_y y) \quad (1)$$ Primary acoustic radiation force

where

$$\Phi = \frac{\kappa_o - \kappa_p}{3\kappa_o} + \frac{\rho_p - \rho_o}{2\rho_p + \rho_o} \quad (2)$$ Acoustic contrast factor

and a is the particle radius, Φ is the acoustic contrast factor, $k_y = 2\pi / \lambda$ is the wave number, E_{ac} is the acoustic energy density, y is the distance from the wall, κ_p is the isothermal compressibility of the particle, κ_o is the isothermal compressibility of the fluid, ρ_p is the density of the particle and ρ_o is the density of the fluid. The model has later been corrected and expanded by Bruus et al. to take viscous and thermal effects into account [18, 19] and new models have been developed to describe the acoustic forces on inhomogeneous fluid compositions and fluid-fluid interactions [20, 21]. For a more comprehensive description and derivation of the PRF model used within this thesis, an excellent introduction is given in [22].

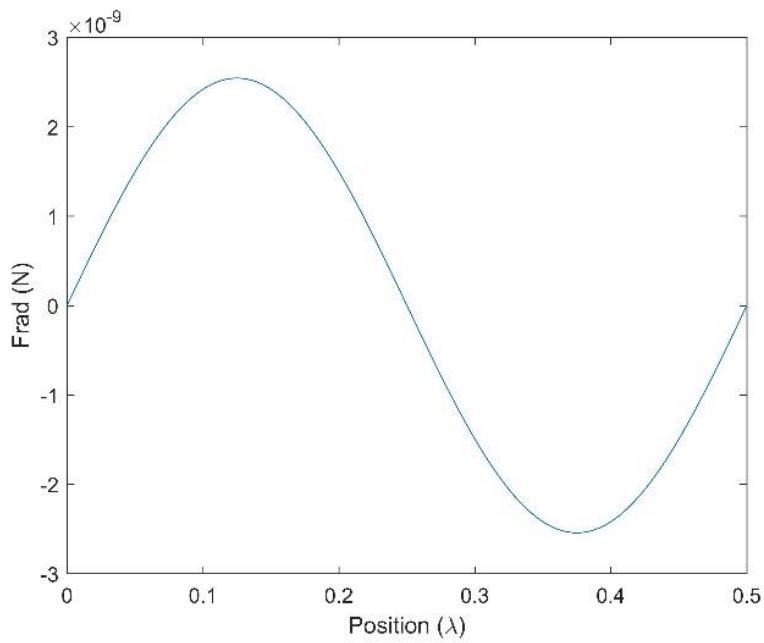


Figure 6. The acoustic primary radiation force in a 2MHz ½ wavelength resonator, as a function of the position. The PRF (F_{rad}) is calculated for a 10µm polystyrene microparticle suspended in water, assuming an acoustic energy density of 1kJ/m^3. A positive PRF indicates a force to the right, and a negative force towards the left in the figure.

The primary acoustic radiation force moves particles into acoustic pressure nodes or antinodes according to the acoustic contrast factor (ACF), Φ, of the particles (see fig. 6 and 7). The contrast factor is derived from differences in the density and compressibility of the particles and the surrounding medium. For suspended solid spheres, such as polystyrene microbeads in water, the largest contribution to the acoustic contrast factor arises from differences in the compressibility, see table 1.

Particle/cell	*Density* $kg\ m^{-3}$	*Compressibility* $10^{-10}\ Pa^{-1}$	*Acoustic contrast factor (suspended in water)*	*Acoustic contrast factor (suspended in PBS)*
Polystyrene	1050	*2,16*	0,193	0,187
Red Blood cell (RBC) [23]	1099	*3,31*	0,124	0,114
Prostate cancer cell line (DU145) [24]	1018	*4,24*	0,031	0,019
Breast Cancer cell line (MCF7) [25]	1068	*4,22*	0,049	0,0358
Water (20 °C)	998	*4,58*	N/A	N/A
PBS (25 °C)	995	*4,38*	N/A	N/A

Table 1. Acoustic properties for different types of cells and particles, and their calculated acoustic contrast factors suspended in water or PBS.

For cells, which typically has a compressibility and density similar to their surroundings, both parts of the acoustic contrast factor must be taken into consideration. Cells suspended in a cell medium or isotonic buffer such as Phosphate Buffered Saline (PBS) typically have a positive contrast factor, and will focus into the pressure nodes of the standing wave. In comparison, particles that are less dense (and more compressible), than the medium, such as lipid vesicles, will focus into the pressure antinodes due to having a negative contrast factor. F_{rad} scales linearly with the contrast factor and the volume, a^3, of the particles.

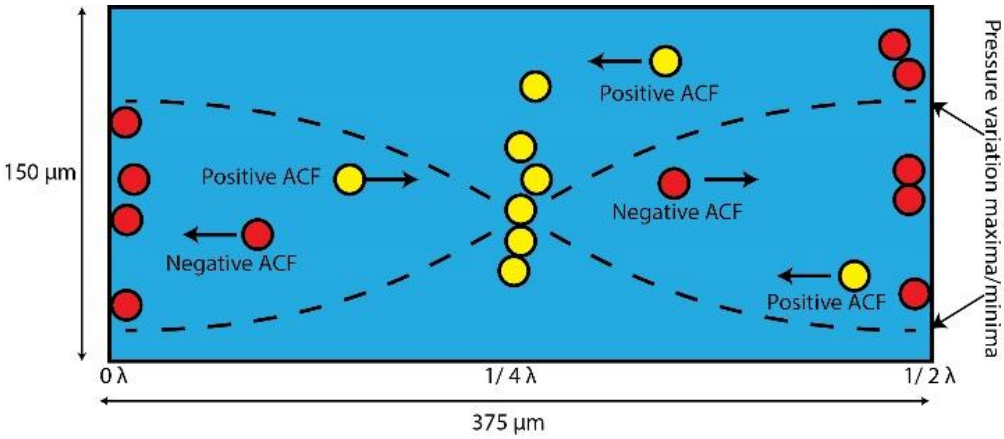


Figure 7. The effect of the primary acoustic radiation force on particles with positive (yellow) and negative (red) contrast factor. The dashed line indicate the pressure variations of the standing wave. The indicated dimensions are typical for the acoustofluidic devices used within this thesis (2MHz ½ λ resonators)

In an acoustic standing wave resonator, the acoustic energy density can become sufficiently high to generate a force (F_{rad}) which results in an observable movement of a particle. As a particle is moved through a liquid, a counteracting force from the Stoke's drag F_{drag} will counteract this movement. When a particle has reached its terminal velocity u_{rad}, the primary radiation force will equal the drag force such as

$$F_{rad} = F_{drag}$$

Using this relationship, the terminal velocity of a particle can be described as:

$$u_{rad} = \frac{2\Phi}{3\mu} a^2 E_{\mathrm{ac}} k_y \sin(2k_y y)$$

where μ is the viscosity of the fluid.

As seen above, u_{rad} is proportional to the diameter squared, and to the acoustic energy density, contrast factor, and wavenumber (frequency). In fig. 8 the velocity of a 10µm diameter polystyrene (PS) microparticle in ½ wavelength standing wave is plotted as a function of the position. Fig.9 shows acoustic focusing of 6µm PS microparticles in one of the acoustofluidic devices used in this thesis.

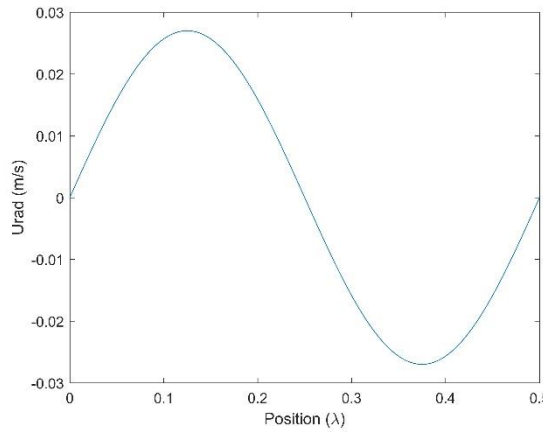


Figure 8. The velocity (u_{rad}), of particle (acoustic streaming not accounted for) in a 2MHz ½ wavelength resonator, as a function of the lateral position. The velocity is calculated for a 10µm PS microparticle suspended in water, assuming an acoustic energy density of 1kJ/m^3. A positive velocity indicates a movement to the right, and a negative velocity towards the left in the figure. It can be seen that the maximum velocity occurs at 1/8 and 3/8 of a wavelength, corresponding to ¼ and ¾ of the channel width.

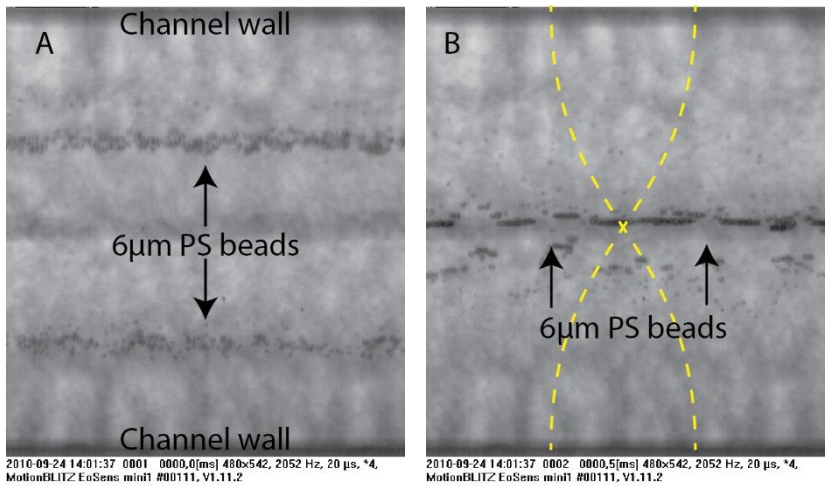


Figure 9. Acoustic focusing of 6 µm polystyrene (PS) microbeads in stopped flow conditions as seen through the top of a microfluidic channel. (A) An image of the beads in their original position, before the acoustic actuation is activated. (B) An image of the beads after ~500µs of acoustic actuation. The majority of the beads have been displaced approximately 80µm towards the center of the channel. The yellow dashed line illustrate the acoustic standing wave.

Acoustic streaming

Actuation of an acoustofluidic resonator at resonance will lead to acoustic streaming (fluid vortices). Several types of acoustic streaming exist, of which Rayleigh streaming has the most impact on cell and particle manipulation in acoustofluidic devices (used in this thesis). This phenomenon was early studied by Lord Rayleigh and has been named thereafter [26]. The model has since then been expanded by Shlichting [27] and several others. An excellent introduction to acoustic streaming is given by Ohlin et al. [28].

In the theoretical model for Rayleigh streaming, the vortices are induced by dissipation of acoustic energy in the viscous boundary layers of the channel walls, parallel to the wave propagation. The acoustic streaming becomes especially pronounced when the height of the channel is smaller than the wavelength of the standing wave.

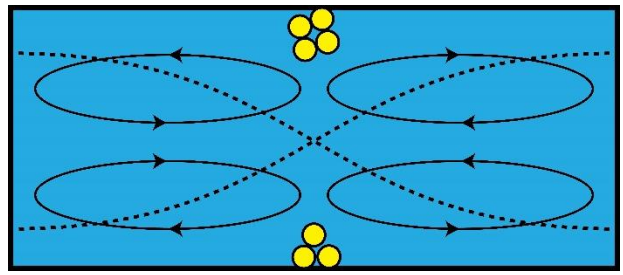


Figure 10. Rayleigh streaming seen from a cross-sectional perspective in an acoustofluidic standing wave resonator. The arrows indicate the direction of the streaming rolls, and the dashed lines line the pressure maxima/minima of the standing wave. The yellow circles indicate possible steady state positions for particles with positive contrast factor.

The acoustic streaming rolls will induce a drag force on particles that are suspended in the channel, which has several implications on the use of particle manipulation within the microfluidic channel (see fig.10). First, the equilibrium positions of a particle is determined by the combination of acoustic forces and streaming induced drag forces. For a particle with a positive contrast factor, the particle will first be focused towards the pressure node by the PRF, and the streaming rolls will then transport the particle perpendicular to the standing wave propagation and flow direction, until the two forces balance each other out [29]. Second, as the drag force scales with radius of a sphere, and the acoustic radiation force with the volume of a sphere, this implies a critical size limit for manipulation of particles using the acoustic radiation force [28].

The acoustic streaming can be suppressed by several means, such as changing the channel geometry, introducing an acoustic resonance in a second dimension[30], or by using fluids with different contrast factor gradients [31, 32]

Different types of acoustofluidic devices

In the research field of acoustofluidics, primarily two types of acoustic resonators are used. Bulk acoustic wave (BAW) and surface acoustic wave (SAW) devices. Essentially, both types of devices rely on ultrasound and acoustic forces to achieve spatial manipulation of cells and particles in microfluidic structures. In practice, the major differences between these two types of devices is the actuation method, and the fabrication material. BAW devices are typically fabricated in hard materials such as silicon, steel or glass. These materials typically have a low damping coefficient and high difference in acoustic impedance compared to water, which facilitates acoustic resonances. BAW devices are usually actuated by a piezoelectric element such as a PZT attached to the structure (see fig. 11). All of the acoustofluidic devices used in papers 1-4 within this thesis can be defined as BAW resonators.

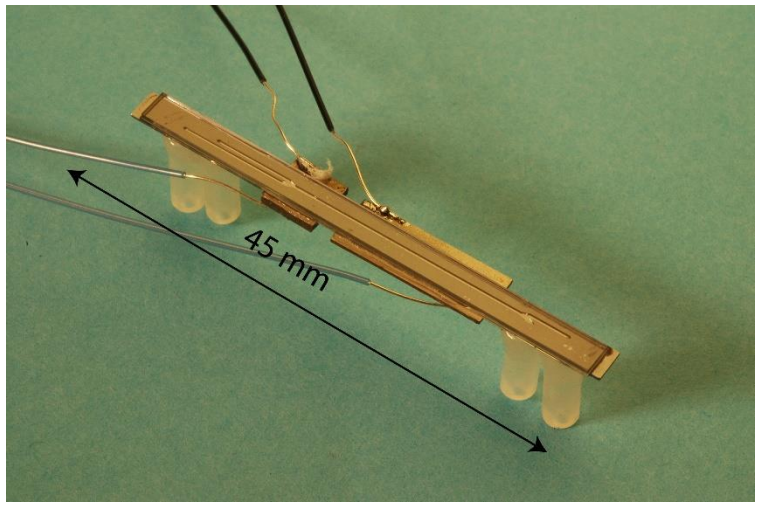


Figure 11. A photograph of one of the acoustofluidic (BAW) devices used in this thesis. The channel is etched in silicon and bonded to glass as a lid. Two piezoceramic plates are attached to the silicon with soldered cables for electrical connection.

SAW devices on the other hand, are often fabricated in soft materials such as Polydimethysiloxane (PDMS), on top of a piezoelectric material. These devices are actuated by integrated interdigitated transducers (IDT). SAW devices has the ability to operate at higher frequencies compared to BAW devices, due to the IDTS as actuation source. SAW devices can be designed for standing wave acoustic manipulation (Standing Surface Acoustic Waves, SSAW), but also for traveling surface acoustic wave manipulation (TSAW) of objects and fluids[33].

3 Conventional Flow Cytometry

Introduction, history and definitions

Flow cytometry is a technology that over the last decades have had a substantial impact on the level of healthcare and understanding in medicine. The definition of cytometry can be derived from the two words cyto, "cell", and metri, "measure" (genitive singular of metrum). Cytometry is the measurement of cells and their properties, and since cells often are suspended in a liquid, these measurements can naturally be performed by having the cells flowing past a detector. Flow cytometry can be performed using a number of techniques, where electrical and optical measurements are the most common methods.

It should be noted that in biomedicine, the term "flow cytometry" is not generally used as a generic term for any type of cytometry method performed in a flow, but is reserved for a laser based technique that measures the optical properties of cells, which in turn may be used for estimating size, morphology and even the phenotype of the cells. Other cytometry techniques that are performed in flow, such as coulter counting, impedance cytometry and deformability cytometry are often termed after their working principle (the measured physical property), although they also technically fall under the term flow cytometry.

From a historic perspective, the field of flow cytometry is not a modern invention. The first flow cytometry device for counting cells was proposed already in 1934 by Andrew Moldavan [34], and in 1948 the technology was demonstrated by C. Lagercrantz [35]. At this time, this technique was named Pulse Cytophotometry, and it was not until 1976 that scientist agreed on using the name flow cytometry. Even though the "pulse cytophotometry" method had been disclosed 20 years earlier, flow cytometry did not have any clinical significance until the late 1950s, when the coulter counter became commercially available.

Wallace H Coulter is often considered as one of the most important inventors in the history of flow cytometry and cell sorting. In 1953, Coulter was granted a patent [36] on the coulter principle, which is a method that relies on electrical measurements to accurately determine the size (volume) of a cell. Around this time, the field of electronics was developing rapidly and applications such as inkjet printing was emerging. The inkjet printer works by periodically ejecting small droplets of fluid (normally ink) from a nozzle at high rates, and these droplets can be spatially manipulated by an electric field. In 1965 Mack Fulwyler successfully combined the inkjet technology with the coulter principle to form the backbone of a new method revolutionizing research in cell biology [37]. The method relied on

the coulter principle to detect the size of different cells, and the inkjet printing principle to sort them into different containers. Using this method, Fulwyler was successfully able to separate mammalian erythrocytes from lymphoma cells, and the world's first "electrical cell sorter" had seen the light of day.

Soon after the use of coulter counters had been adopted in clinical laboratories, it was realized that size measurements alone were not enough to distinguish between different cell types, and optical measurements could provide additional information about the cells. By illuminating cells in a flow with a collimated light beam, the scattered light at different angles from the cell could be collected and used for "fingerprinting" different cell types based on their optical properties. Absorption methods was originally used in the optical flow cytometers, but in 1968, fluorescence microscopy had gained a widespread use in biological research, and this functionality was built into a flow cytometer by the German company Partec. This instrument would be the predecessor to the modern flow cytometer. In 1974, the American company Becton, Dickinson and company (BD) combined the optical flow cytometry technology with the inkjet printing sorting method, just as Fulwyler had done with the coulter principle in 1965, and the first Fluorescence Activated Cell Sorter (FACS) was made commercially available. Still today, BD and Partec are two of largest companies supplying flow cytometers and FACS instruments to the market. Flow cytometry and cell sorting has become a critical enabling technology within biological research and medicine.

Unfortunately, some companies have named their flow cytometer models with the term "FACS", eg BD "FACSCanto", even though these instruments lack the cell sorting capability. As a result of this, the terms "FACS analysis" or "FACS data" are often misused for flow cytometry data. It should be remembered that all flow sorters (FACS instruments) are flow cytometers, but not all flow cytometers are FACSes (even though they have the term FACS in the model name)!

Over the last decade, a field within the microfluidic research community called micro-flow cytometry (μ-cytometry) has been gaining attention. This research field focuses on the miniaturizing of flow cytometers, cell sorters and their components. In order to evaluate the progress of this field in terms of potential and limitations, it is important to understand how the conventional flow cytometers are constructed. A review of the μ-cytometry field is available in chapter 4 of this thesis.

The Coulter Counter

The coulter counter is an instrument that counts and measures the size of cells. The instrument is using the coulter principle, which is an electrical measurement. A typical coulter counter consist of two chambers, which are joined by a small orifice or aperture slightly larger than the cells or particles that are measured (fig. 12). The

sample has to be suspended in a conductive medium, typically an electrolyte suspension, as a small current is driven between the two chambers. Due to the constriction created by the orifice, the majority of the voltage drop and hence the gradient of the electrical field is concentrated to the orifice. Cells typically have a different conductance compared to the electrolyte (lower), and when the sample is flowing through the orifice, the cells will constrict the flow of the electrical current and produce a change in the voltage over the orifice. This change in impedance is proportional to the volume of conductive electrolyte displaced by the cell in the orifice.

If the flow is known through the orifice, the concentration and thus the exact number of cells can be measured in a sample. The coulter counter is able to count thousands of cells every second, and is typically used in medicine for blood analysis (complete blood counts), where the number of red and white blood cells is an important indicator of a patient's health.

The coulter principle can be implemented in a number of design formats, which all have advantages and disadvantages. A common design of the coulter counter is the aperture format, where a small orifice is created in the wall of a glass tube. When the sample is aspirated from a larger beaker through the glass tube, all cells have to pass through the aperture and generate a measureable signal. Although many different designs have been developed over the history of the coulter counter, the major advancements in the recent decade has been in the detection circuitry, with better electronics and noise suppression, allowing a quicker and more accurate analysis of a sample. Impedance cytometers and microfluidic impedance cytometers are built on the coulter principle, but has the ability to measure the electrical impedance at different frequencies for further information on the analyzed cells.

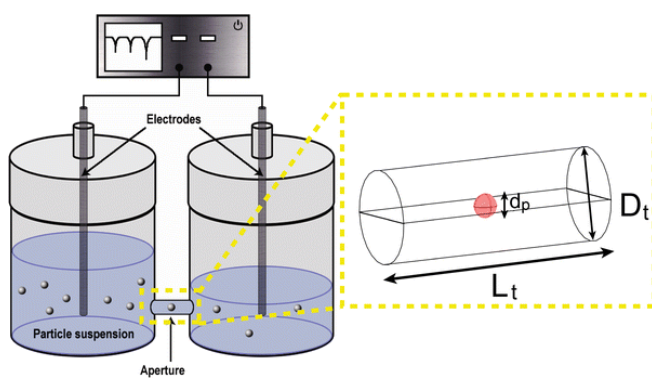


Figure 12. An illustration of the coulter counter working principle. Reprinted from reference [38] with permission from Springer.

Flow cytometry

An (optical) flow cytometer can be seen as a microscope with a very high throughput. Instead of an image, a flow cytometer is generating a set of numbers

which are the measured parameters of a cell. These parameters can be both intrinsic and extrinsic, meaning that they can be measured with or without the use of reagents (often called probes). By combining the multiple measured parameters of an individual cell, which is similar to a fingerprint, the specific cell type can often be identified (with some additional information about the sample). Setting up the criteria for identification of a cell type in flow cytometry is called gating, a process that can be adapted for most cell types.

A flow cytometer has the ability to measure more than 100000 cells per second, making it much faster than a conventional microscope for analyzing and quantifying a complex sample. This high analysis rate is possible since the cells flow very fast through a focused laser beam. The measurements are derived from the light scattered from the cells, collected at different angles and different wavelengths. This ability to look at hundreds of thousands of cells per second makes the flow cytometer ideally suited for quantification of different cell types within a sample. Flow cytometers can be bought from several different suppliers, over a wide range of prices (from approx. 30000 USD and up) depending on capability.

A modern flow cytometer typically consist of the following components:

- A fluidic pumping system. Pressure driven systems are most common, but peristaltic pumps exist in some models (e.g. BD Accuri A6).
- A flow cell, which aligns all cells in the flow prior to analysis. The standard method for focusing cells is hydrodynamic focusing. The Invitrogen Attune flow cytometer is using acoustic focusing to improve the focusing of the cells.
- A light source. Most cytometers have at least two or three lasers operating at different wavelengths.
- Detectors and analog to digital (AD) converters. Typically PMTs (photomutiplier tubes) are used as the detectors.
- A computer, controlling all the different components.

In fig. 13, a simplified illustration shows the general principle of flow cytometry. The sample containing the cells to be analyzed is injected into the flow cell, and is hydrodynamically focused to a thin stream by the sheath fluid. The flow cell typically has a co-axial symmetry for the flow focusing. The flow focusing is performed in order to center the cells in the laser beam, which is typically in the range of ~20μm width, and the sample stream, also called core stream, should be much smaller than this. The flow focusing is critical for a correct analysis, since the light beam typically has a Gaussian intensity profile. The light scattered from the cells is collected at two angles, forward of the laser, and perpendicular to this angle. The intensity of the Forward scattered light (FSC) is used as an indicator of the cell size. The laser light is blocked by an obscuration bar on the other side of the flow

cell and does not hit the FSC detector directly, only the light scattered at small angles is collected.

The side scatter detector(s) (SSC) are placed at a 90° angle to the laser beam. The light scattered at this angle is an indicator of granularity of the cells. The fluorescence emission is also collected from this beam path. Dichroic mirrors are used to split up the light beam into different wavelengths and is collected by filtered PMTs. Most cytometers have 2-5 detectors per laser on the SSC beam path. These are called fluorescence channels, often labeled FL1,FL2 etc..

Experimentally, the instrument status (flow focusing, laser alignment etc.) is verified by running a sample through the cytometer containing calibration beads with a known homogenous size and amount of fluorophore. The coefficient of variation (CV) of the scattered light is used as an indicator of the instrument status (preferably <1%). The electrical readout from the PMT is converted by AD converters, and presented in real time on the computer connected to the instrument. The data is often presented in 2D, so called "dot plots" with the scatter signal from two of the detectors on the respective axes.

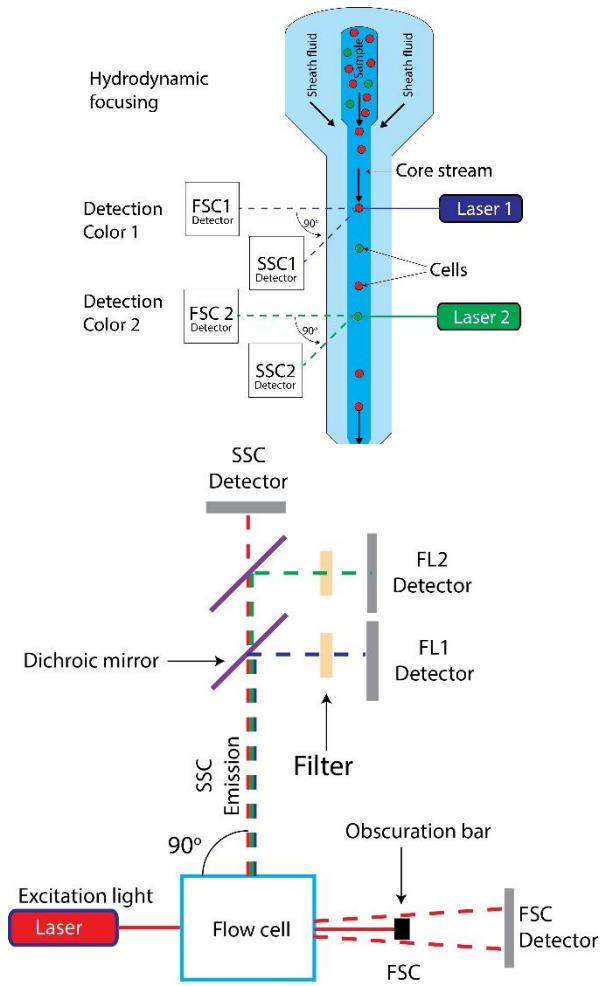


Figure 13. A simplified illustration of the working principle of a flow cytometer.

Flow sorting (FACS)

The fluorescence activated cell sorter (FACS) is a natural evolution of the flow cytometer. A FACS is essentially a flow cytometer equipped with cell sorting capability (based on the inkjet principle). The basic principle of a conventional FACS is illustrated in fig. 14. Much of the design is very similar to a flow cytometer. At the end of the flow cell, a nozzle with a vibrating piezo is periodically breaking up the stream into tiny droplets (in air). The cells that have been analyzed upstream are encapsulated in these droplet. These drops are passing through an electric field induced by two charged plates (deflector plates). By controlling the charge of the generated droplets, the droplets can be deflected by the electric field and thus their trajectory is controlled. Finally, the droplets are collected in different collection tubes.

The charge is added to the individual droplets by charging the entire liquid column using an electrode in contact with the liquid. Just as the drop has broken off from the main stream, the charge of the column is reverted to neutral and a new charge can be added to the next drop before it breaks off. Depending on the nozzle size, normal drop generation rates for a modern flow sorter is 20-150 kHz. As the charge of each droplet has to be addressed individually, this requires fast electronics and precise knowledge of the droplet breakup point. Also stable droplet generation is essential for the process to be robust. Most FACS instruments are using a camera and a stroboscopic light source to monitor the drop generation, in order to find the correct timing parameters between charging of the liquid column and the droplet breakup point. Fig. 14 shows the drop generation from a BD FACSAria2 as displayed in the BD FACSDiva software.

The charge set to each individual drop is determined by the scattered light collected in the flow cytometry module upstream of the sort module, and the user defined logic gates for the sort process. Again, the timing between the laser interrogation point and the drop breakup point is essential for accurate sorting.

When performing flow sorting, all cells entering the flow cell will be encapsulated in droplets, but not all droplets will contain cells. This is because the droplet generation is a periodic process, while cells entering the flow cell is a stochastic process. The flow cytometer can calculate with high probability in which drop a cell will be encapsulated and add a desired charge. As the droplet then is sorted as a "package", it is preferred that the droplets does not contain more than one cell per drop. There is a tradeoff between the throughput in cells per second and the purity of a sort [39]. In general, a higher drop generation rate leads to a higher purity or throughput. The drop generation is limited by the size of the orifice in the drop generation nozzle, where a smaller nozzle is capable of generating drops at a higher rate. Unfortunately, smaller nozzles are more prone to clogging, a fact which is well described by Howard Shapiro's "First Law of Flow Cytometry: A 51µm Particle CLOGS a 50 µm Orifice" [40].

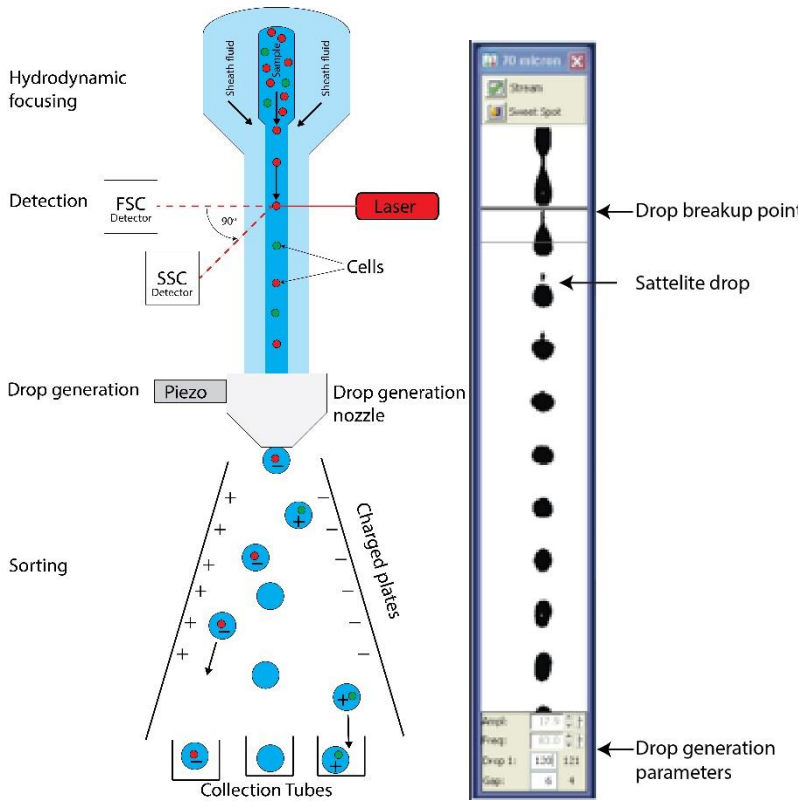


Figure 14. A simplified illustration of the working principle of a conventional FACS instrument. The droplet generation is imaged with a stroboscopic light source and the image is analyzed in real time for correction of timing parameters related to the sort process.

4 Microfluidics for cytometry and cell sorting

Introduction

Over the last decade, major advances have been made in the field of μ-cytometry. This research field focuses on the miniaturizing of flow cytometers, cell sorters and their components. In general, a μ-cytometer is built on the same principle as a conventional cytometer, but with miniaturized and more integrated unit operations/components. Furthermore, a microfluidic cell sorter (μFACS systems) is a μCytometer with sorting capability.

The most important functions/unit operations a fully operable μ-cytometer requires are:

1. Handling of and pumping the sample and other liquids through the device.
2. Focusing the particles into a well-defined stream past the detector
3. A detection system to analyze and classify the cells. This is often based on optical or electrical signals

A fully functional μFACS requires all the above specified functions, plus a sorting capability, which means some mechanism of deflecting/manipulating cells at an individual level, based on the detected signal. This also requires the detector and signal processing function of the sorter to be able operate in real-time in order to provide the sort decisions required for the sort mechanism.

In the literature, a few papers can be found describing fully functional μ-cytometers and μFACS systems. Several papers separately describe advances made in one of the above mentioned unit operations, without being integrated into a fully functional system. This chapter gives a brief introduction to different μ-cytometry methods. A more extensive review is given on recent advances in methods for spatial manipulation of cells (flow focusing and sorting), intended for cytometry applications. Finally, a few functional μFACS systems are reviewed. Methods for detection methods and signal processing are well reviewed in references [41-43].

μ-Cytometry methods in research

4.1.1 Optical μ-cytometry

Optical μ-cytometers measures the optical properties of cells, just as the conventional flow cytometer. In a conventional cytometer, FSC, SSC, and fluorescence intensity are the traditionally measured parameters of a cell. The scattered light is normally collected by lenses outside the flow cell and focused to optical fibers coupled to beam splitters and PMTs. Measuring both the FSC and

SSC signals from a μ-cytometer fabricated on a planar microchip is slightly different from the conventional cytometer, as the scattered light is not readily accessible for collection by lenses due to the geometry of the microfluidic chip. Instead, integrated optical fibers [44-46] or chip integrated optical waveguides and lenses [47, 48] are commonly used. Oakey et al. demonstrated a μ-cytometer capable of analyzing 25000 particles/ second [49] using FSC and backscattered light.

Approaches have also been made based on measuring other optical parameters of cells, such as Raman spectra [50] and fluorescence lifetime [51], although with modified conventional cytometers. Holmes et al. demonstrated a μ-cytometer with confocal optical detection, capable of analyzing 250 particles/s [52]. Optical measurement methods can also be complemented with electrical measurements [53] for additional information from a cell (fig. 15).

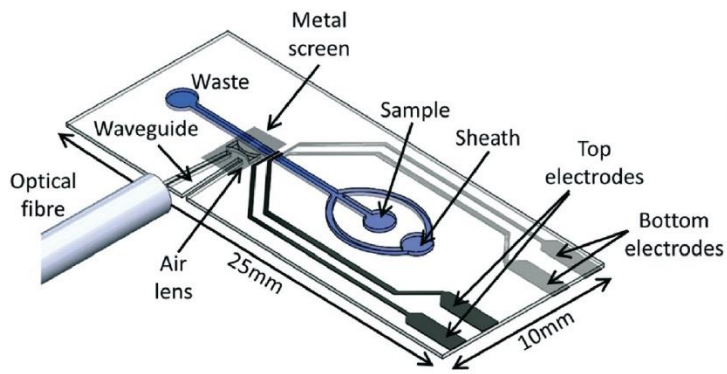


Figure 15. A microfluidic cytometer with combined impedance and optical detection. Reprinted from reference [53] with permission from the Royal Society of Chemistry

4.1.2 Impedance μ-cytometry

The traditional coulter counter senses the displaced coulter volume, and thus the volume of the measured cell. By measuring the impedance between two (or more electrodes) at multiple frequencies, additional information about the cells in between can be acquired.

In an early work, Gawad et al. presented an impedance based μ-cytometer [54], capable of analyzing cells at 100 cells/s. By using an opposing electrode configuration (see fig. 16) for increased sensitivity, Holmes et al. [55] demonstrated an impedance based μ-cytometer capable of leukocyte differentiation at a throughput of approximately 12 cells/s. Other works describe different advances made in the field [56, 57]. Recently, Grenvall et al. presented a novel impedance cytometer [58] combined with acoustic focusing. Typical flow rates for impedance based μ-cytometers is in the range of a few μL/ min. The sensing aperture in most impedance μcytometers is typically a few particle diameters wide for increased sensitivity, but inherently limits which sample sizes and flow rates that can be used.

Efforts have been made to create coulter counters with dynamic aperture size [59], using non-conductive sheath flows.

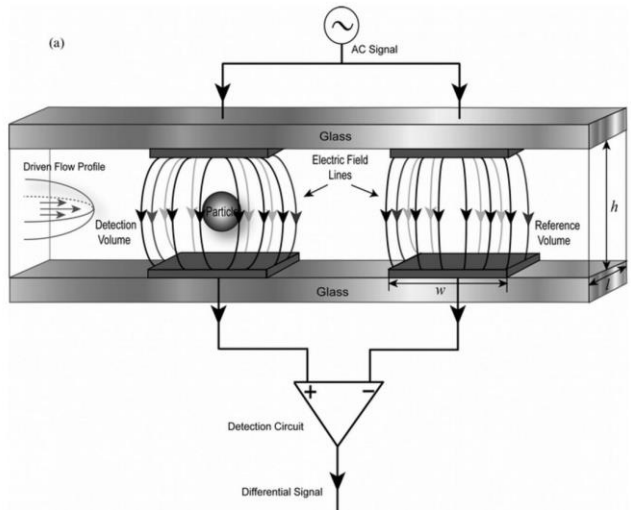


Figure 16. The working principle of an impedance cytometer with opposing sensing electrodes. Reprinted from reference [60] with permission from the Royal Society of Chemistry.

4.1.3 Other cytometry methods

Imaging cytometry is a relatively new technique in the cytometry field, and is based on imaging cells in a flow. Commercially available imaging cytometers are available from a variety of companies. These cytometers are essentially conventional flow cytometers equipped with imaging capability (imaging is triggered by the FCS/SSC signal). Imaging cells which are traveling at high linear velocities require extremely short shutter times, and/or special image acquisition techniques. Most commercial imaging cytometers are using specialized CCDs with dynamic shutter functions and image deconvolution algorithms to acquire sharp images of cells. A few microfluidic imaging cytometers have been proposed: Zmjan et al, demonstrated an imaging cytometer [61] using acoustic focusing to put all cells in the focusing plane of the camera.

Deformability cytometry is a type of imaging cytometry that measures the deformability of cells for mechanical phenotyping (fig 17). Cells can be deformed by a variety of methods such as physical constrictions[7, 62] , acoustic forces [63], optical forces[64] , or hydrodynamic forces [65]. Normally, deformability cytometry is computationally heavy and requires the analysis of images to be performed off-line. Recently, image processing techniques have become fast enough

for on-line analysis of the acquired images, allowing real time mechanical phenotyping of cells a rate of 100 cells/second [66].

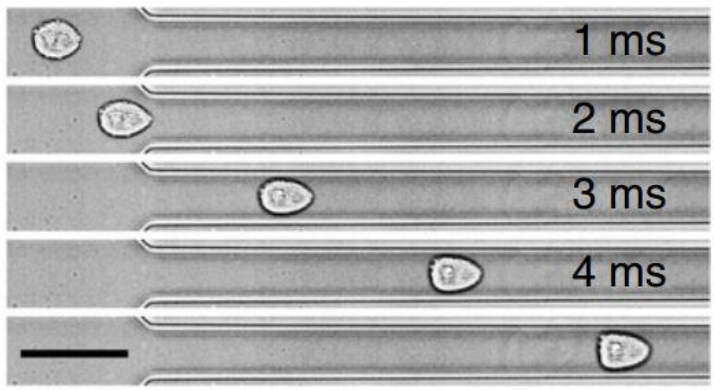


Figure 17. An image series from a deformability cytometer (research prototype), showing the deformation of a cell flowing through a constriction. Reprinted from reference [66] by permission from Macmillan Publishers Ltd: Nature.

Cell and particle alignment for µ-cytometry

Depending on the cytometry method (electrical or optical), alignment of the cells to be analyzed in the flow can be of high importance. There are two main reasons why focusing of the cells is important. First, due to the parabolic flow profile of a pressure driven flow, unfocused cells will travel through the system with different velocities, and thus have different retention times in the system. This might affect the measured properties of a cell, especially if the collected signal is time integrated. Different velocities do also produce timing errors if several detectors (e.g. laser illumination spots) are used, and makes downstream sorting of the cells very difficult.

Second, the collected signal is often dependent on the position of the measured cell, depending on the cytometry method used. For example, an optical cytometry system is using a focused laser beam to illuminate the cells. The illumination spot often has a Gaussian intensity profile [40], with makes the intensity of the scattered light and thus collected signal highly position dependent. Impedance cytometers with planar electrode configurations are also sensitive to position irregularities of the measured cells, because of the difficulty in producing a homogenous electrical field [54, 58]. When using most flow cytometers, particle alignment is first experimentally verified by running a bead suspension (calibration beads) with homogenous size and fluorescence intensity.

4.1.4 Definitions used for cell alignment in flow cytometry

Cell alignment/focusing for flow cytometry can be done in one or more dimensions. Currently, there is no consensus in the definitions used by researchers in

microfluidic community. In this thesis, the following definitions are used (see fig. 18):

No alignment/focusing: Particles are not confined in any dimension within the microfluidic structure, and can as a result be present anywhere.

1-dimensional alignment: Particles are confined in one dimension, and randomly distributed in the other two dimensions. Particles can be anywhere in a plane.

2-dimensional alignment: Particles are confined in two dimensions, and free to move in the third dimension. Particles can be anywhere on a line. In flow cytometry, the flow is parallel to the third "free" dimension in order to transport particles to the detector.

3-dimensional alignment: Particles are confined in all three dimension, and not free to move anywhere. As particles cannot be transported by the flow to the detector, this type of alignment may not be practical for flow cytometry.

In many microfluidics papers, the term 3-dimensional focusing is used to describe the same type of alignment as what is defined as 2-dimensional focusing in this thesis.

Finally, there is another aspect of pre-alignment beyond the 3-dimensional position, which must be considered when performing flow cytometric analysis of non-spherical cells (such as red blood cells and sperm cells) [40]. In an optical flow cytometry system, the scattered light from a non-spherical cell is highly dependent on the cells relative orientation to the incident light beam. As a result, two identical cells passing the detector with different orientations might produce very different light scattering signals, confounding the identification process. Currently, few cytometry systems have the ability to control the orientation of non-spherical cells.

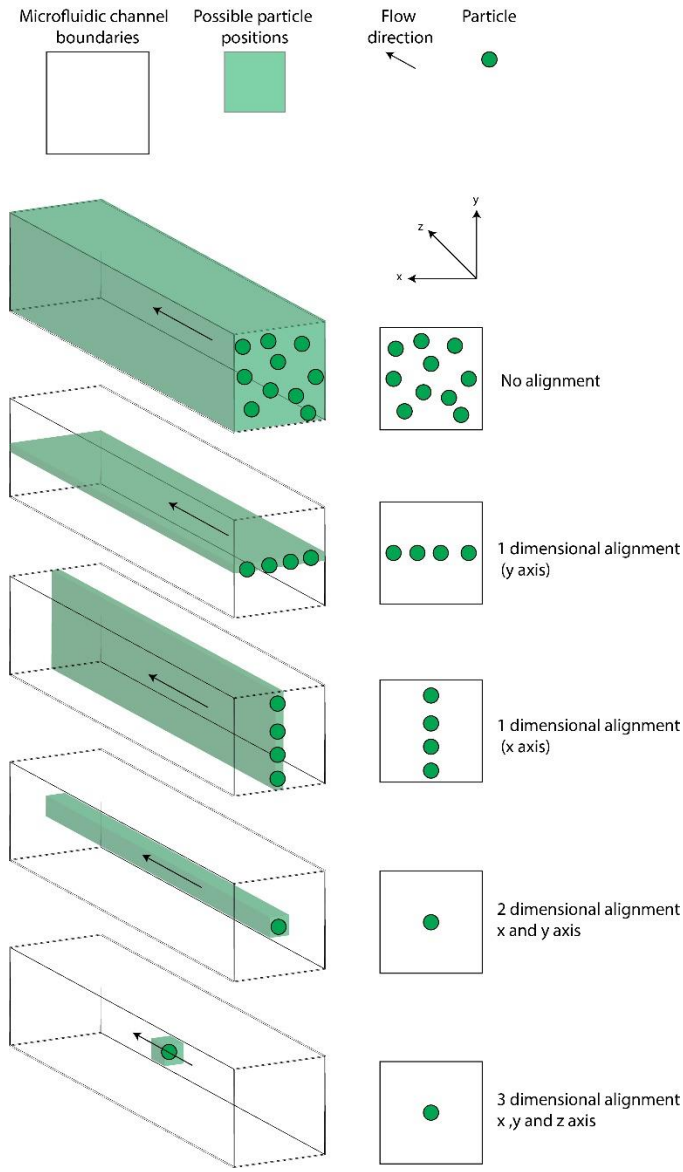


Figure 18. An illustration of the different types of particle alignment and corresponding definitions used within this thesis.

4.1.5 Hydrodynamic focusing

Hydrodynamic focusing is the most common method of aligning particles in a flow. By laminating the sample stream with a second fluid (sheath flow), the sample can be confined into a thin stream[12]. The width of the sample stream is practically controlled by the ratio between the sample and sheath flow. In conventional flow cytometers, a structure with coaxial symmetry (essentially a small tube in a large tube) is used to achieve 2-dimensional hydrodynamic focusing of the sample.

Microfluidic chips on the other hand, are most of the time designed with a planar and layered layout in mind. As fabricating a coaxial symmetry on a planar substrate is challenging, coaxial designs are abandoned in favor of other more feasible approaches.

In a microfluidic chip fabricated on a planar substrate, 1-dimensional hydrodynamic focusing is straightforward to achieve in the horizontal plane by using a trifurcated inlet. 1-dimensional focusing does not produce a uniform velocity distribution of a sample, which is required for many cytometry applications. 2-dimensional focusing yields a uniform velocity distribution and can be achieved in microfluidic chips. Using hydrodynamic focusing, sheathing the sample both in the horizontal and vertical dimensions typically requires more complex microchip designs and fabrication techniques. Several microfluidic solutions exist for hydrodynamic 2-dimensional focusing. One approach is to fabricate multiple layers in a manifold that compose the final microfluidic component [67-70], or to implement complex geometry variations[71] in one layer. Less complex microchip designs [72, 73] has also been shown to produce 2-dimensional sheathing of a sample. It should be noted that a complex microchip design is not always an obstacle for commercial success, as long as the structure can be mass produced using cheap mass fabrication techniques such as injection molding.

Hydrodynamic focusing has the benefit of keeping the sample and sample suspension liquid away from the walls of the microfluidic channel, which is reducing fouling of the walls in the channel, especially important in the region of the channel where the detection is carried out. In contrast to active field-based methods (such as acoustophoresis), hydrodynamic focusing acts on the sample suspension fluid rather than on the individual sample entities. This is beneficial as it removes the lower size limit normally associated with field based methods, making it possible to focus samples with sizes down to the molecular level.

The drawback of hydrodynamic focusing is that the sheath fluid is diluting the sample (and accelerating the core stream), which can result in extended sample acquisition times. It might also be impractical to use hydrodynamic focusing in a handheld device since it requires a second fluid container containing the sheath fluid. In an attempt to remove the necessity of having a sheath fluid, it has been shown that air can be used to achieve hydrodynamic focusing [74].

4.1.6 Inertial focusing

Inertial effects in microfluidics can be utilized for particle and cell manipulation in a wide number of applications [8, 75]. Towards flow cytometry applications, cell focusing, sorting and enrichment operations are of particular interest [8, 49]. Inertial microfluidics is a method that does not require any externally applied fields, forces or fluids.

The main force(s) behind inertial focusing which makes a particle shift position in a flow can be derived from the laminar flow conditions at low (but non-zero) Reynolds numbers. More specifically, a lift force exists (the wall effect), which pushes a particle (during flow) away from the walls in a microfluidic channel. A second counter-directional force exists due to shear effects from the parabolic flow profile, and counteracts this force, see fig 19. The equilibrium position of a particle is found when these two forces are balanced. Depending on the specific system, the equilibrium position of a particle is determined by a number of parameters, where particle size, viscosity of the fluid, channel geometry, and flow rate are among the important parameters. Typically, the equilibrium position is away from the cross-sectional center of the channel, and several equilibrium positions exist. By adding a curved geometry to the channel layout on the microfluidic chip, further manipulation of equilibrium positions is possible [44, 76].

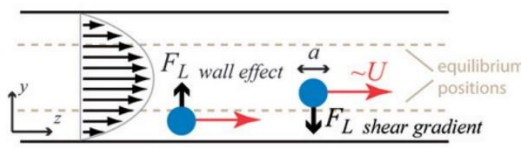


Figure 19. The two main forces responsible for focusing particles to an equilibrium position. Figure reprinted from reference [75] with permission from the Royal Society of Chemistry.

The main benefit of using inertial focusing is that it is "passive" in a sense that it does not require any externally applied fields or sheath flow, making it feasible for massive parallelization. The drawbacks on the other hand are that the equilibrium position of a focused cell is dependent on its size, and other experimental parameters that the user might want to vary in order to optimize the system as a whole. Also, inertial focusing systems generally are using microfluidic channels with small cross-sectional areas, which increases the risk of clogging.

Inertial focusing has been shown to affect the intercellular distances when many cells are present in the microfluidic channel [77-80], see fig 20. Controlling the intercellular distances, which are normally considered random when using other types of focusing techniques, is highly beneficial for both detection and sorting efficiency, and has the potential to increase the performance of a cytometer or sorter drastically. Delivering cells with regular intercellular spacing to the detector (and sorting mechanism) reduces the probability of detecting and sorting "doublets", which is when two or more cells are being detected simultaneously and cannot be distinguished.

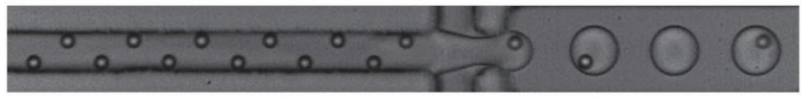


Figure 20. Inertial ordering of particles and subsequent encapsulation in droplets. Figure reprinted from reference [75] with permission from the Royal Society of Chemistry.

4.1.7 Dielectrophoretic focusing (DEP)

By applying an alternating electrical field across a microfluidic channel, cells and particles within will experience a force directed towards or away from the electrical field gradient. The electrical field is generated from electrodes placed in or next to the channel.

The dielectrophoretic force F_{dep} [81] acting on a particle is generally described as:

$$F_{dep} = 2\pi\varepsilon_m r^3 Re[K(\omega)]\nabla E_{rms}^2$$

where r is the radius of the sphere, E_{rms} is the root mean square value of the electric field, and $K(\omega)$ is the Clausius–Mossotti factor. The Clausius-Mossoti factor is determined by the difference in complex permittivity (polarizability) of the object and the surrounding medium, according to:

$$K(\omega) = \left(\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}\right)$$

$$\varepsilon^* = \varepsilon - \frac{j\sigma}{\omega}$$

where ε_p^* and ε_m^* are the complex permittivity of the object and the surrounding medium.

Depending on the difference in polarizability between the specific cell and suspension buffer, the force is either positive or negative. Typically a negative DEP force is desired as it repels the cells from the electrodes, which are placed at the channel boundaries (walls or top/bottom). DEP can be used as a standalone method (see fig. 21) or in complement with e.g. hydrodynamic focusing to achieve 2-dimensional sample focusing for different cytometry applications [82-85].

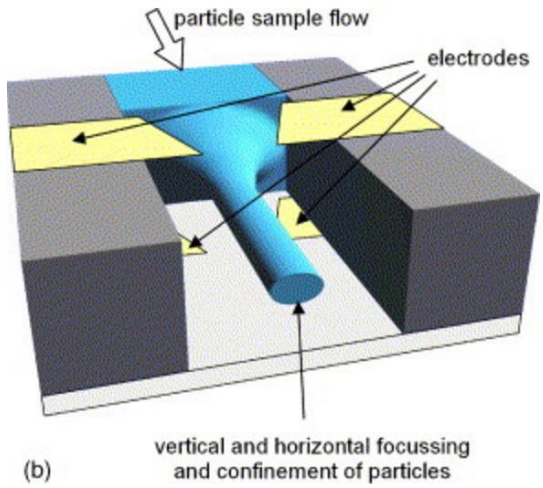


Figure 21. DEP 2-dimensional focusing of particles for flow cytometry. Figure reprinted from reference [82] with Permission from Elsevier.

In general, DEP forces are weaker in comparison to magnetic or acoustic forces, which limits the throughput of a cytometry system. Similarly to these other types of field based manipulation techniques, the force is volume dependent with a practical lower size limit of approximately 1μm. It also requires buffer conditions that are slightly different from normal cell culture mediums, which require additional sample preparation protocols.

4.1.8 Acoustic focusing

Acoustic forces have been used to spatially manipulate cells towards cytometry applications in a number of ways. Currently there are two types of acoustic focusing methods being used, bulk acoustic waves (BAW) and surface acoustic waves (SAW).

BAW has been shown to be effective for 1 and 2-dimensional focusing in a variety of devices with different geometries. One approach of using BAW focusing is in micro-capillaries made of glass or metal. Using a cylindrical metal capillary, Goddard et al. demonstrated 2-dimensional focusing [86-88] of mammalian cells. A coaxial geometry has the benefit of being able to focus cells in 2 dimensions using a single resonance mode and actuation frequency[89]. The downside of using a cylindrical geometry for focusing of the sample, is that the sample stream must be transferred into a channel with rectangular cross-section to allow light scattering measurements to be carried out. Using a rectangular glass capillary, Graves et al. demonstrated 1-dimensional focusing into multiple, even-spaced streams[90], towards parallelized flow cytometry.

BAW focusing can also be implemented in microchannels fabricated on planar substrates. Fabricating a rectangular channel on planar substrate is a straightforward

process, and allows simple splitting and recombination of multiple fluid streams, in contrast to a capillary based approach. Using rectangular channels etched in silicon, 1-Dimensional focusing has been demonstrated [90, 91] for single and multi-node focusing (fig. 22) of a sample.

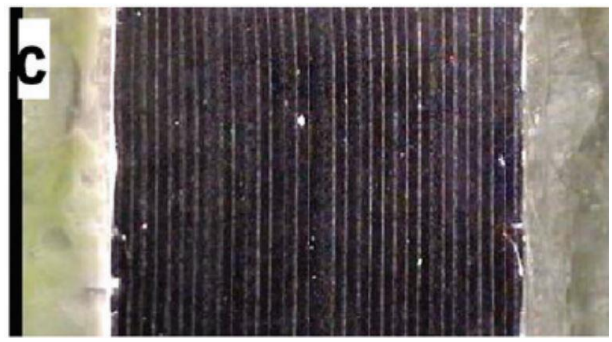


Figure 22. Multinode acoustic focusing of 10μm polystyrene particles into 33 different sample streams. Figure reprinted from reference [91] with permission from the American Society of Chemistry (Copyright 2012).

By actuating an acoustic resonance in a second dimension, typically the vertical dimension, 2-dimensional focusing for flow cytometry has been shown in microchannels etched in silicon [92] (square geometry), and in glass [58] (isotropic etch geometry). Depending on the aspect ratio of the cross-section of the microchannel, 2-dimensional focusing can be achieved using single, or multiple actuation frequencies. In paper 1 of this thesis, 2-dimensional acoustic focusing is demonstrated using a single actuation frequency. In paper 2, 2-dimensional acoustic focusing is achieved in an isotropically etched glass chip using two actuation frequencies. The typical actuation frequencies used in BAW devices are between 0.5-5MHz.

In contrast to BAW methods, standing surface acoustic wave (SSAW) methods are used for particle focusing in devices where the microchannel is fabricated in a soft material, such as PDMS. Using SSAW, 1-dimensional [93] and 2-dimensional focusing [94] has been demonstrated. SSAW devices are typically fabricated in PDMS, bonded to a Lithium Niobate $LiNbO_3$ substrate. The SAW devices are actuated by interdigital transducers (IDTS), which comprise of patterned metal stripes deposited on the $LiNbO_3$ substrate. IDTS are effective over a higher frequency range, giving SSAW device the ability to operate at much higher frequencies. In theory, several benefit are expected from operating at high frequencies. However, SSAW devices has only been demonstrated working at low flow rates, typically in the range of a few μL/min.

Cell orientation

The relative orientation of a non-spherical cell to the incident light beam is essential for a correct flow-cytometric analysis [40, 95]. Typical non-spherical cells are red blood cells and sperm cells. While several techniques exist for pre-aligning the position of cells, few techniques exist for controlling the orientation. It was early realized that controlled orientation was necessary to identify the sex (chromosomal DNA quantification) of a mammalian sperm cells using flow cytometry. One of the first successful attempts to orient sperm cells for flow cytometry was demonstrated by Dean et al. [96]. In their work, hydrodynamic focusing with an asymmetrical geometry on the flow focusing nozzle was used to create identical orientation from cell to cell. However, this approach required that the tail was separated from the head of the sperm cell to be efficient.

Using inertial effects, Di Carlo et al. demonstrated rotational self-ordering of red blood cells in a 50 μm wide channel [8]. In this device, RBCs were oriented to project their smallest area (edge-on orientation) to the sensor, with their longest dimension parallel with the flow vector. Using a different approach, Beech et al. demonstrated that RBCs could be oriented to project their largest area (face-on orientation) to the sensor [7]. This was accomplished by reducing the depth of a channel to less than the diameter of the RBCs and thus constrain the cells to only one orientation. The downside to using a very shallow channel depth is that it limits the maximum flowrate, and the device becomes more prone to clogging or delamination.

Optical traps have also been shown to be able to control the orientation of non-spherical entities [97, 98]. This technique is more suited for precise control of the orientation of a single cell, and is difficult to apply to continuous flow systems such as flow cytometers.

In paper 2, controlled orientation of RBCs in a continuous flow is demonstrated using 2-dimensional acoustophoresis.

μFACS systems and cell sorting techniques.

Just as the conventional FACS is a flow cytometer with sorting capability, a microfluidic sorting device (μFACS) is a μCytometer with sorting capability. In the literature, a few reports exist that describe fully functional μFACS devices. Other works can be found that separately demonstrate a sorting mechanism, without any cytometry capability integrated with the device. Naturally, the information from the cytometer part of a FACS is needed in order to make a sort decision, and by definition, "real sorting" is not demonstrated in these papers. Instead, this type of reports typically present data on how fast a continuous stream of particles can be periodically directed between two or more outlets. In this thesis, these papers are

reviewed separately from fully functional sorters, and the performance of a standalone sorting mechanism and a fully functional sorter should not be compared.

Theoretically, any type of technique that has the ability to redirect an individual cell or small part of a fluidic volume from one trajectory (e.g. outlet of a microfluidic chip) to another, can be used for flow sorting. For example, most field based particle manipulation techniques that have been demonstrated for sample focusing, can also be used for sorting purposes. Most field based microfluidic sorting techniques relies on an actuation principle with a variable field strength, acting directly on the cells and controlling their trajectory through the device. Field based techniques with dynamic field strength may also act on the sample suspension liquid rather than on individual cells to achieve sorting. Finally, some approaches that can be used for sorting are purely hydrodynamic, such as mechanical micro-valves and electro-osmotic flows.

4.1.9 Comparing performance between sorters

The key performance parameters that should be considered when comparing different sorters are throughput, recovery and sort purity. All of these parameters are interdependent, and the overall performance of a sorter is generally limited by how fast the sort mechanism can operate (see supplemental information of paper 1). In some reports, post-sort enrichment is reported instead of purity of the sorted sample. The enrichment is the ratio between the sample purity, pre- and post-sort. The post-sort purity can be calculated from the reported enrichment for comparison purposes (as long as the start purity is reported).

4.1.10 Sorting methods

In this section, standalone sorting mechanisms that have been demonstrated in devices without cytometry capability are highlighted. The reported throughput is often the theoretical maximum of the proposed sort mechanism, and should not be compared directly with the throughput of a fully functional sorter.

Several field based techniques have been shown to be capable of directing cells, particles or droplets between different outlets of a chip. Electrophoresis has been shown to be feasible for binary sorting of cells [99], and DEP has been shown to be capable of directing cells between two [100] or more outlets [101]. Throughput was not reported in these studies. DEP has also been shown to be capable of droplet sorting [102], at a throughput of 100 drops/s.

SSAW based techniques have been reported for binary [103] and multi-outlet sorting [104], as well as droplet based sorting [105]. Recently, advances in minimizing the active IDT focusing area has made sort frequencies in the kHz range possible [106, 107].

Among the valve based technique, Chen et al demonstrated a piezoelectric actuated sorting mechanism with response times between 0.1-1ms [108]. Their device was based on a PZT, displacing a membrane in fluidic contact with flow in the channel, deflecting particles, see fig 23. This type of technology have been implement in the wolf cell sorter, commercialized by Nanocellect Biomedical Inc.

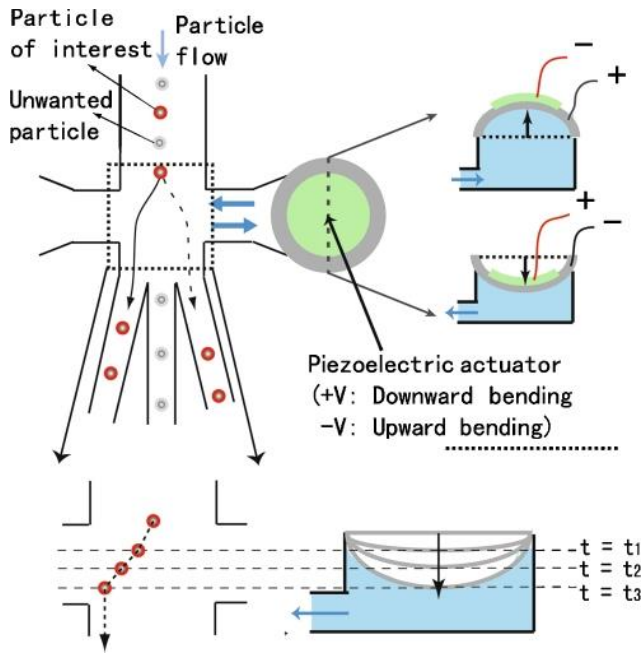


Figure 23. A sorting method based on a piezoelectric actuation of a membrane, displacing fluid in the sort region of a microfluidic channel. Figure reprinted from reference [108] with permission from Springer.

4.1.11 Fully functional sorters

Several reports exist that describe fully functional microfluidic sorters. So far, none of the proposed devices demonstrate key performance capabilities that are on par or better than a conventional FACS. Despite this, the μFACS systems have a few advantages compared with conventional flow sorting, especially when it comes to sorting of very specific samples [109]. A commonly emphasized advantage of a μFACS (that is not based on the inkjet principle) is that the sorting is aerosol free. Aerosol free sorting is beneficial when sterility is of importance, but also when the sample is hazardous. These sorters also have the ability to be integrated with additional unit operations downstream, although this still remains to be demonstrated. Another commonly stressed fact is that conventional flow sorting damages cells. Although this argument is valid for some specific cell types, the conventional FACS is suitable for sorting of most cell types if the sorting parameters are correctly tuned (e.g. pressure and drop generation rate). Despite being slower than the conventional FACS systems, the performance of reported μFACS systems

has generally improved a lot over the last decade. Major advances have been made both with field-based sorters, and valve-based sorters.

Among the valve based sort techniques, Fu et al. [110] presented a sorter with chip-integrated pneumatic micro-valves in 2002. Using this approach, up to 44 cells/s could be sorted. In 2003, Wolff et al. demonstrated sorting of beads and chicken blood using fast external micro-valves with a response time of 2.5 ms. In this work, a throughput of 12000 cells/s was reported, but the sort purity was very low as a consequence of the high throughput. In 2010, Cho et al. demonstrated sorting of mammalian cell at a throughput of 1500 cells/s [111, 112] with high purity. This device was based on PZT-actuated integrated membranes, capable of deflecting particles between three outlets.

A variety of field based techniques have been reported for flow sorting. In 1999, Fu et al. [113] demonstrated a field based technique acting directly on the fluid in the sort channel. Using electro-osmotic flows, sorting of GFP expressing *E. coli* bacteria in a micro-fabricated soft polymer device was achieved. In 2009, Baret et al. [114] demonstrated high purity droplet sorting at a throughput of 300 drops/s. In this work di-electrophoretic forces (DEP) was used to gate fluorescent droplets between two outlets. Optical forces can also be used for sorting. In 2004, Wang et al. [115], achieved binary sorting of mammalian cells at a throughput of 100 cells/s, using a focused laser beam for on-demand deflection of cells.

A few μFACS systems based on acoustic forces can be found in the literature. In one of the earliest works using acoustic forces for flow sorting, Johansson et al. [116] demonstrated sorting of fluorescent microbeads at a throughput of ~20 beads/min. In this work, fluids with different acoustic contrast factors were used to facilitate the sort process. Paper 1 in this thesis describes flow sorting of fluorescent microbeads at a throughput of 150 beads/s. More recently, Navaz et al. [117] presented a fully functional sorter based on SAW, capable of sorting 1200 events/s with high purity (>90%), see fig 24.

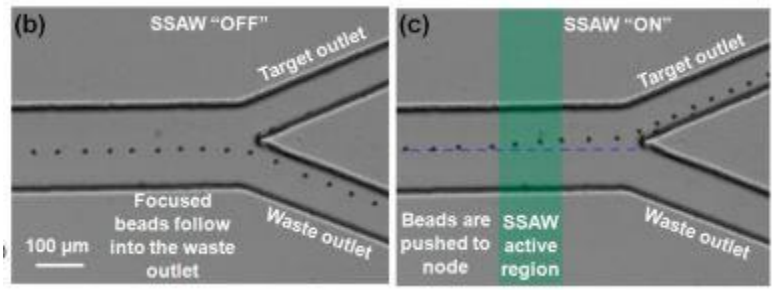


Figure 24. A microfluidic sorter based on SSAW technology. Upon detection of a fluorescence signal, the IDTS are actuated and beads are deflected towards the target outlet. Reprinted from reference [117] with permission from the American Society of Chemistry.

Microfluidic technology in commercial cytometers and sorters

Conventional flow cytometers have matured over more than 50 years and is a tool used in most biomedical research today. In the last decade, a new type of flow cytometers has entered the market, not necessarily with increased performance, but instead with a lower cost and a smaller footprint in the lab. These cytometers are often called benchtop cytometers, and their footprint is approximately equal to that of an office laser printer. These cytometers are also possible to bring along and use in the field [118], although they cannot be considered as fully portable.

A very common statement in the literature is that microfluidics and the LOC concept offers the tools for shrinking the footprint of the cytometers even further, to that of a handheld device. In order to assess the possibilities and challenges of such statement, it is important to remember that the conventional flow cytometer is already based on microfluidic technology. The majority of the fluidic operations takes place in the flow cell, which in a conventional cytometer has a footprint of only a few centimeters. Most of the bulk in the instrument comprises of fluidic containers and pumps, lasers, optics and detectors, circuitry etc. Simply miniaturizing the fluidic handling parts may not result in a significantly smaller instrument, instead miniaturization and integration of all parts in the instrument is essential.

Currently, handheld coulter counters are available from Beckman Coulter, and Cambridge Biosciences offers a handheld flow cytometer called Moxi Flow, although with limited capabilities. Also, the Attune acoustic flow cytometer from

Thermo Fisher (previously Life Technologies) comes from research in the microfluidics area.

There has been a call from the consumer side for disposable flow cells for flow cytometers. Currently, the flow cell of a conventional flow cytometer is expensive since it is fabricated in quartz glass using advanced machining methods, and is therefore naturally not disposable. Instead of being disposed, extensive cleaning procedures are being used to clean and sterilize the flow cell between samples or experiments. Sterilization is extra important when performing cell sorting. In the Sony SH800 cell sorter, the flow cell has been replaced with a disposable planar plastic microfluidic chip, which is the result of research in the microfluidics area.

Since 2014, Milteny Biotech is offering a microfluidic cell sorter (MACSQuant Tyto) to the market, capable of binary sorting of cells. The device is using a magnetic micro-valve capable of 30000 valve actuations/s according to the manufacturer. A recent review by Shields et al. [119] gives a comprehensive overview on the efforts and challenges of commercializing microfluidic cell sorters.

4.1.12 A few reflections on different sorting techniques

So far, the inkjet technology based droplet sorting mechanism used in the conventional FACS has superior performance in comparison to most μFACS systems. The inkjet principle relies on a static field, and achieves sorting by changing the property (electric charge) of the individual drops. As adding or removing charge on a drop can be done extremely fast, this technology's sorting rate is limited by how fast the drops can be generated, which practically is in the range of hundreds of kHz.

Many μ-FACS systems presented in the literature are using a dynamic field acting directly on the cells (where the field strength is varied according to sort decisions) to achieve sorting. It is likely that these dynamic field based sorting techniques may never be able to compete with the ink-jet principle in terms of sorting throughput. As a field-induced force acts on all particles within the field (a volume), only one cell can be present at a time within this volume (for single cell sorting). Further, a target cell needs to be deflected a minimum distance orthogonally to the flow in order to be sorted from non-target cells. The sorting speed in these systems is ultimately limited by the time required to deflect a particle the minimum distance required (a velocity), before a new sort event can take place. Naturally, there is a limit on how fast a particle can be dragged through a liquid before the shear stress becomes too high for the cell to maintain membrane integrity.

A field based sorting mechanism such as acoustophoresis, acting directly on a cell or particle, where the force on the cells is depending on their intrinsic properties, may also not be suitable for the sorting of cells with very different sizes or acoustic contrast factors. On the other hand, these entities may then instead be separated by

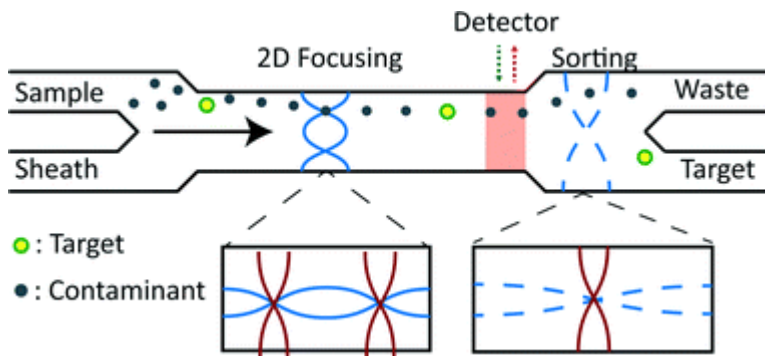
their intrinsic properties, which typically can be achieved at much higher throughput as compared to sorting. The acoustic FACS presented in paper 1 could be used as cytometer with separation capability. Field based techniques may also not work well on very small cells such as bacteria, due to the volume dependence of the force. This limit might be alleviated by encapsulation in droplets, with a larger volume for the force to act upon.

5 Summary of papers

Paper 1

Acoustic actuated fluorescence activated sorting of microparticles

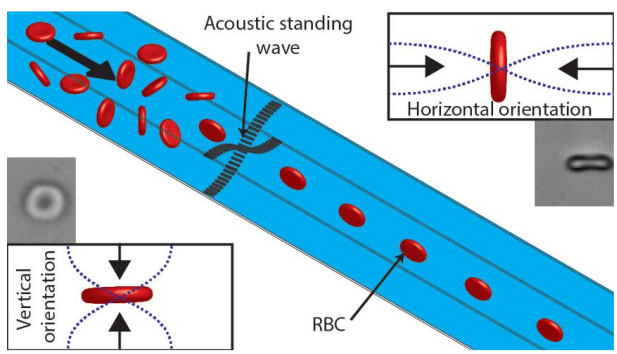
This paper describes a fluorescence activated sorter realized in a BAW acoustofluidic chip. Sorting is performed by deflecting a desired particle with a short acoustic burst, triggered by fluorescence intensity detection. The system utilizes two-dimensional acoustic pre-focusing driven by a single actuation frequency, and 10µm polystyrene particles could be two-dimensionally focused at flow rates up to 1.7 mL/min. Particles could be sorted based on their fluorescence intensities at throughputs up to 150 particles/s. The highest purity reached was 80% (starting at a pre-sort purity of 20%) when sorting at an average rate of 50 particles/s. The average recovery of a sort was 93.2 ± 2.6%. The presented system enables fluorescence activated cell sorting in a continuous flow microfluidic format that allows aseptic integration of downstream microfluidic functionalities, opening for medical and clinical applications.



Paper 2

Continuous Flow Two-Dimensional Acoustic Orientation of Non-spherical Cells

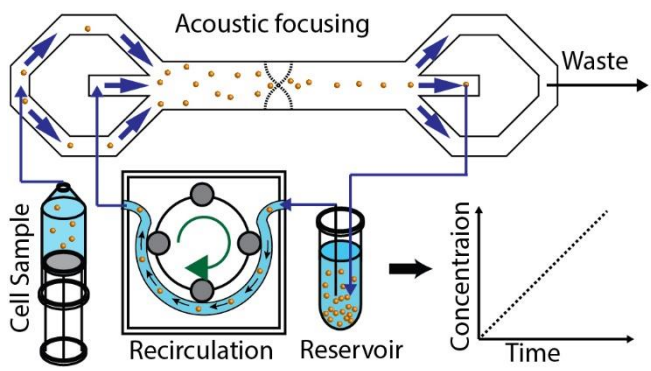
Non-spherical cells, such as red blood cells or sperm cells, pose a challenge when performing flow cytometric analysis. The uncertainty of a non-spherical cell's relative orientation to incident light beam produce large intensity variations in the scattered light, which interfere with the analysis of these and other cell populations in the same sample. This paper presents an acoustofluidic chip utilizing ultrasonic standing waves to focus and orient red blood cells two-dimensionally in the channel center. The cells can be oriented to show either their flat (face-on orientation) or up-ended side (edge-on orientation) toward the optical axis and the observer. In an acoustic standing wave field the cells will be rotated until the direction of the smallest dimension is parallel with the direction where the acoustic energy is strongest. While keeping the cells focused in the channel center, utilizing acoustic resonances in two dimensions, the orientation can be controlled by increasing the acoustic energy in either the horizontal or vertical resonance mode. It was shown that 87.8 ± 3.8% of the red blood cells could be horizontally oriented while 98.7 ± 0.3% could be vertically oriented.



Paper 3

Thousand-Fold Volumetric Concentration of Live Cells with a Recirculating Acoustofluidic Device

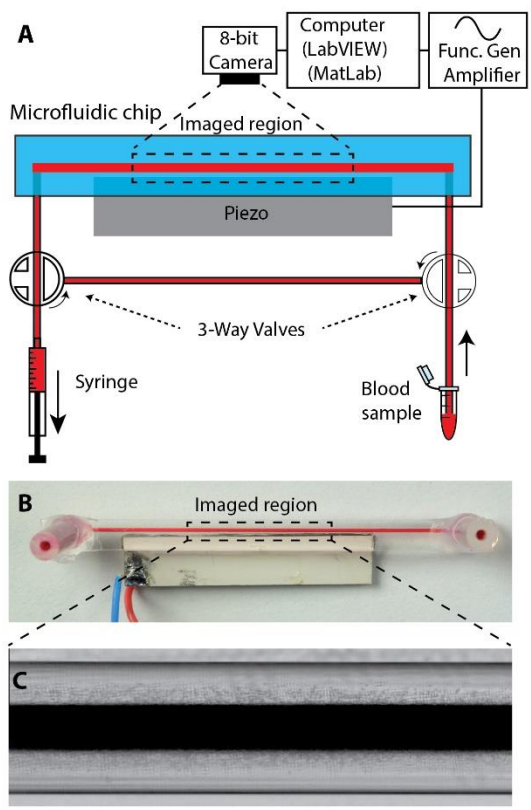
The ability to concentrate cells from dilute samples into smaller volumes is an essential process step for most biological assays. Volumetric concentration is typically achieved via centrifugation, but this technique is not well suited for handling small number of cells, and is difficult to automate. This paper presents a device that combines acoustofluidics with a novel recirculating architecture to achieve >1000-fold enrichment of cells in a label-free manner, at high volumetric throughput (500 µL/min) and with high recovery (>98.7%). The device can be used with a wide variety of different cell types and a viability test indicate that this concentration strategy does not affect cell viability. The device could be readily adopted to serve as a "sample preparation" module that can be integrated with other microfluidic devices to allow analysis of dilute cellular samples in large volumes.



Paper 4

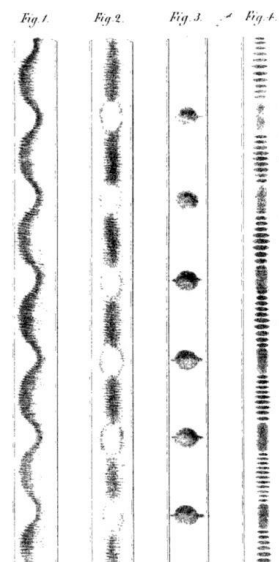
Rapid hematocrit level measurement in an acoustofluidic device

The hematocrit level (HCT) in blood is the relative volume of blood cells to the total volume of a blood sample, and is an important indicator of a patient's blood status. HCT measurements of blood from patients, blood donors and athletes are routinely performed every day. Measurements can be performed using centrifugation, which is difficult to automate, or in larger centralized labs using whole blood analyzers, which is expensive. This paper presents an alternative and simple method to measure the HCT levels in blood samples, using acoustofluidics. Whole blood is acoustophoretically focused in a microfluidic glass channel, and the channel is imaged using an 8-bit camera. From the image, the relative area of the blood cells to the channel area of the glass chip gives an HCT value with linear relationship to the centrifugation based method. The presented device is capable of providing an accurate HCT measurement in less than 30 seconds.



6 Populärvetenskaplig sammanfattning

Ljud är egentligen vibrationer i ett material, som kan vara i fast, flytande eller i gasform. Det mänskliga örat kan fånga upp och omvandla vibrationer inom frekvensområdet 20 Hz till 20 kHz (20-20000 svängningar per sekund), till nervsignaler vilka vår hjärna tolkar som ”ljud”. Frekvenser som ligger över det mänskliga hörbara området kallas för ultraljud. En vibration, vilket normalt beskrivs som en sinusformad fram-och-tillbaka-gående rörelse, kan användas för att generera en kraft, och därmed också en nettoförflyttning av ett objekt. Detta fenomen observerades redan på 1600-talet av Robert Hooke, som såg att små partiklar utstrött som ett pulver på en metallplatta ansamlades i vissa mönster när man vibrerade plattan med en fiolsträng. Fenomenet utforskades ytterligare av bland annat den tyska fysikern August Kundt som observerade att dammpartiklar till synes ”svävade” och ansamlades periodiskt i orgelpipor och glasrör när de ljöd (se fig. 25) [120].



Figur 25. Ansamling av dammpartiklar i orgelrör, så som observerat av August Kundt år 1866. Figuren är hämtad ifrån August Kunds Publikation [120] i Annalen der Physik, 1866 med tillstånd ifrån John Wiley and Sons. (Figure reprinted from reference [120] with permission from John Wiley and Sons.)

På samma sätt som Kundt kunde förflytta och ansamla dammpartiklar i luft, kan ultraljud användas för att påverka och förflytta celler och små partiklar suspenderade i en vätska. Mikrofluidik-kanaler är små kanaler (ungefär lika stora som ett hårstrå)

som man kan leda vätska igenom. Genom att vibrera en mikrokanal fylld med blod, vid ca 2 MHz (2 miljoner svängningar per sekund), kan man till exempel ansamla de röda blodkropparna till vissa områden i kanalen, och på så sätt separera blodceller och blodplasma ifrån varandra.

Blod innehåller mest röda blodkroppar, men även andra celltyper. För diagnos av och forskning på sjukdomar så är det ofta av intresse att plocka ut specifika celltyper och bakterier ur en blandning av celler, och utföra vidare tester av dessa. Eftersom celler är små, ca 10μm (en hundradel av en millimeter) och inte är synliga utan mikroskop, så är de svåra att hantera och sortera individuellt med manuella verktyg så som pincetter och nålspetsar. Dessutom så finns det väldig stora mängder av vissa celltyper i blod, medan andra är mycket sällsynta. I en droppe blod finns det till exempel ca 5 miljoner röda blodkroppar, men ungefär bara en bakterie ifall patienten har en allvarlig infektion. För att exempelvis kunna bestämma vilken typ av antibiotika som skall användas så måste bakterien hittas och isoleras. Naturligtvis är det opraktiskt att med mikroskop och nål sitta och gå igenom 5 miljoner blodkroppar för att kunna hitta den önskade bakterien.

Ett sätt att lösa detta problem är att använda ultraljudet som en slags osynlig pincett, vilken kan användas för att plocka ut vissa specifika celltyper ut till exempel blod eller en annan blandning av celler. Genom att låta en programmerad dator eller annan elektronik, kopplade till en kamera eller ljussensor, styra ultraljudet, kan celler av rätt typ hittas och isoleras med höga hastigheter.

I denna avhandling beskrivs olika sätt att med hjälp av ultraljud manipulera olika lösningar av celler. Bland annat så beskrivs ett sätt att sortera ut flourescensmärkta (färgade) celler ifrån omärkta celler, med en hastighet av 200 celler per sekund med hjälp av ultraljud. Vidare beskrivs en metod att kontrollera icke-sfäriska cellers orientering inför en kamera eller ljussensor. Genom att använda ultraljud visas också en metod att samla upp och koncentrera en liten mängd celler ifrån en större volym (>1dl i biomedicinska sammanhang) till en mindre och mer hanterbar volym för kommersiella analysinstrument (ca 100μl). Till sist beskrivs en metod att med ultraljud bestämma hematokritnivån i en patients blod, något som är viktigt för att upptäcka exempelvis vissa blodsjukdomar eller dopning.

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