LUND UNIVERSITY

Clinical applications of acoustophoresis in blood based diagnostics

Petersson, Klara

2018

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Petersson, K. (2018). *Clinical applications of acoustophoresis in blood based diagnostics* (1 ed.). [Doctoral Thesis (compilation), Division for Biomedical Engineering]. Department of Biomedical Engineering, Lund university.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Clinical applications of acoustophoresis in blood based diagnostics

Klara Petersson

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Engineering, Lund University, Sweden.
To be defended in Belfragesalen D15 BMC, Klinikgatan 32, Lund.
March 23 at 09:15

Faculty opponent
Dr. Stéphanie Descroix

Organization	Document name
LUND UNIVERSITY Department of Biomedical Engineering P.O. Box 118, SE-221 00 Lund, Sweden	DOCTORAL DISSERTATION **Date of issue** February 20, 2018
Author: Klara Petersson	**Sponsoring organizations** Knut and Alice Wallenberg Foundation, Swedish Research Council, Vinnova, European Union Seventh Framework Programme, Crafoord Foundation, SSF Strategic Research Centre, Royal Physiographic Society

Title: Clinical applications of acoustophoresis in blood based diagnostics

Abstract

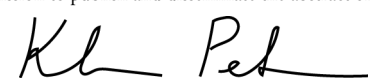
In this thesis, acoustofluidics has been used to process blood samples as a sample preparation step prior to biomarker detection. This is often required as the large amount of blood cells otherwise can interfere with the detection method. Acoustofluidics means moving particles or cells with acoustic forces within microfluidic channels. Ultrasound is used to create a standing wave between the channel walls. By matching of the frequency to the channel with, a half wavelength standing wave can be created between the side walls. Blood cells introduced into this channel will then be focused to the centre pressure node while the plasma can be extracted from the sides. This approach has here been used for three different applications. First it was integrated with a prostate-specific antigen (PSA) immunoassay which not only enabled detection of PSA from whole blood but also resulted in a faster detection because of the continuous flow of produced plasma, constantly bringing new analyte close to the capturing antibodies. Secondly a similar blood plasma separation was integrated with an acoustic trap for enrichment and microchip PCR to enable fast identification of bacteria from sepsis blood samples. In a small clinical study this acoustofluidic based detection system could identify *Escherichia coli (E. coli)* in half of the samples compared to blood culture, probably the ones with the highest bacteria load. In an attempt to improve the bacteria recovery of the blood bacteria separation, another setup where buffer flow in the centre of the channel laminated the blood sample along the sides was tested. By carefully control the dilution of blood sample and the acoustic impedance (density times speed of sound) bacteria recovery was significantly improved. Third, simple and fast haematocrit (HCT) measurements were also achieved in an acoustofluidic device combined with image analysis. The area of the focused blood cells compared to the whole channel area gave a measure with linear relationship to HCT measure by standard methods. This way of measuring HCT could be used prior to the separation step in acoustofluidic sample preparation device. From this I conclude that acoustofluidic sample preparation, which continuously can produce plasma both from diluted and undiluted blood samples, is a promising tool for miniaturised, automated total analysis systems for the detection of rare biomarkers.

Key words: Acoustophoresis, Acoustofluidics, Ultrasound, Microfluidics, Blood, Blood separation, Blood plasma, Lab-on-a-chip, Point-of-care, blood diagnostics

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language:
ISRN: LUTEDX/TEEM – 1112 – SE Report-nr: 1/18	English
ISSN and key title	ISBN: 978-91-7753-590-4 (print) ISBN: 978-91-7753-591-1 (electronic)
Recipient's notes / **Number of pages:** 158	**Price**
Security classification	

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature  Date 2018-02-20

Kaffe, kaffe, kaffe!

-Ellen, 2 years old

Public defence
March 23, 2018, 09.15 in Belfragesalen, D15, BMC, Klinikgatan 32, 222 42, Lund, Sweden

Advisors
Professor Thomas Laurell
Dr. Pelle Ohlsson
Department of Biomedical Engineering, Lund University, Sweden
Professor Johan Malm
Department of translational medicine, Lund University, Sweden

Faculty opponent
Dr. Stéphanie Descroix
Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, CNRS UMR168, Paris, France.

Examination board
Dr. Agnes Michanek
HemoCue AB (Radiometer Group), Ängelholm, Sweden
Professor Anders Christensson
Department of Clinical Sciences, Internal Medicine, Lund University, Sweden
Professor Åsa Melhus
Department of Medical Sciences, Infectious Medicine, Uppsala University, Sweden
Deputy member: Dr. Jason Beech
Department of Solid State Physics, Lund University, Sweden

Chairman
Associate Professor Johan Nilsson
Department of Biomedical Engineering, Lund University, Sweden

Cover illustration
Acoustic focusing of red blood cells. Illustration by Klara Petersson.

ISBN: 978-91-7753-590-4 (print)
ISBN: 978-91-7753-591-1 (electronic)
Report-nr: 1/18
ISRN: LUTEDX/TEEM – 1112 – SE
Printed in February 2018 by Tryckeriet i E-huset, Lund, Sweden

© 2018 Klara Petersson

List of publications

I **Integrated acoustic immunoaffinity-capture (IAI) platform for detection of PSA from whole blood samples**
*A. Ahmad Tajudin, **K. Petersson**, A. Lenshof, A.-M. Swärd-Nilsson, L. Åberg, G. Marko-Varga, J. Malm, H. Lilja and T. Laurell.*
Lab on a Chip, 2013, 13, 1790-1796

II **Integrated acoustic separation, enrichment and microchip polymerase chain reaction detection of bacteria from blood for rapid sepsis diagnostics**
P. Ohlsson, M. Evander*, **K. Petersson***, L. Mellhammar, A. Lehmusvuori, U. Karhunen, M. Soikkeli, T. Seppä, E. Tuunainen, A. Spangar, P. von Lode, K. Rantakokko-Jalava, G. Otto, S. Scheding, T. Soukka, S. Wittfooth and T. Laurell*
Analytical Chemistry, 2016, 88, 9403-9411
*Shared first authorship
ACS Editors' Choice (open access)

III **Acoustofluidic hematocrit determination**
K. Petersson, O. Jakobsson*, P. Ohlsson, P. Augustsson, S. Scheding, J. Malm and T. Laurell*
Analytica Chimica Acta, 2018, 1000, 199-204
*Shared first authorship

IV **Acoustic impedance matched buffers enable separation of bacteria from blood cells at high cell concentrations**
P. Ohlsson, **K. Petersson***, P. Augustsson and T. Laurell*
Submitted manuscript, 2017
*Shared first authorship

Authors contribution

Paper I

Designed, fabricated and optimised operation of plasma separation chip. Performed major part of experiments, including optimisation plasma separation and optimisation of flow based PSA assay. Performed part of the data evaluation and writing.

Paper II

Participated in planning of the integration strategy. Tested different possible approaches for blood plasma separation. Fabricated and optimised blood plasma separation device for more stable and reproducible performance. Tested the blood plasma separation for separation efficiency and bacteria recovery. Was running part of the experiment on the integrated system (separation + enrichment + PCR assay) both when testing with spiked blood samples and when running clinical samples. Participated in discussions concerning data evaluation as well as part of writing.

Paper III

Participated in developing the idea. Participated in the planning of experiments and setup. Performed major part of experiments, major part of data evaluation and major part of writing.

Paper IV

Participated in developing the idea and planning of experiments. Performed major part of the separation experiments. Investigated acoustic impedance of different buffers. Participated in data evaluation and performed big part of writing.

List of abstracts in conference proceedings

I **Integration of acoustophoresis of whole blood with PSA diagnostics on protein microarrays.**
K. Petersson, A. Ahmad-Tajudin, A. Lenshof, A-M. Swärd-Nilsson, L. Åberg, G. Marko-Varga, J. Malm, H. Lilja and T. Laurell.

Poster presented at Medical technology days 2011, October 11-12, Linköping, Sweden.

II **Separation of bacteria from blood cells by acoustophoresis.**
Pelle Ohlsson, Klara Petersson, Andres Lenshof, Ingbritt Åstrand-Grundström and Thomas Laurell.

Poster presented at USW network 2012, September 21-22, Lund, Sweden.

III **Integration of acoustophoresis of whole blood with PSA diagnostics on protein microarrays.**
K. Petersson, A. Ahmad-Tajudin, A. Lenshof, A-M. Swärd-Nilsson, L. Åberg, G. Marko-Varga, J. Malm, H. Lilja and T. Laurell.

Poster presented ay JSPS Symposium 2013, March 26-27,Tokyo,Japan.

IV **Acoustophoresis separation of bacteria from blood cells for rapid sepsis diagnostics**
Pelle Ohlsson, Klara Petersson, Per Augustsson and Thomas Laurell.

Oral presentation at µTAS 2013, October 27-31, Freiburg, Germany.

V **Separation of bacteria from blood cells by acoustophoresis for rapid sepsis diagnostics**
Pelle Ohlsson, Klara Petersson, Per Augustsson and Thomas Laurell.

Poster presented at WAM-nano 2014, June 23-24, Copenhagen, Denmark.

VI **Integrated acoustic sample preparation for rapid sepsis diagnostics.**

K. Petersson, M. Evander, P. Ohlsson, M. Soikkeli, T. Seppä, A. Lehmusvuori, E. Tuunainen, A. Spanger, U. Karhunen, S. Wittfooth and T. Laurell.

Oral presentation at μTAS 2014, October 26-30, San Antonio, Texas, USA.

VII **Acoustic separation of bacteria from blood cells at high cell concentrations enabeled by acoustic impedance matched buffers.**

Pelle Ohlsson, Klara Petersson and Thomas Laurell

Poster presented at μTAS 2014, October 26-30, San Antonio, Texas, USA.

VIII **Acoustic impedance matching enables separation of bacteria from blood cells at high cell concentrations**

Pelle Ohlsson, Klara Petersson and Thomas Laurell

Oral presentation at International Congress on Ultrasonics, Acustofluidics 2015, May 10-14, Metz, France

IX **An integrated acoustic sample preparation system for rapid sepsis diagnostics**

Evander, M., Ohlsson, P., Petersson, K., Mellhammar, L., Lehmusvuori, A., Karhunen, U., Soikkeli, M., Seppä, T., Tuunainen, E., Spangar, A., von Lode, P., Rantakokko-Jalava, K., Otto, G., Scheding, S., Soukka, T., Wittfooth, S., and Laurell, T.

Oral presentation at International Congress on Ultrasonics, Acustofluidics 2015, May 10-14, Metz, France

X **Acoustic separation, enrichment and microchip PCR detection of bacteria from blood**

P. Ohlsson, M. Evander, K. Petersson, L. Mellhammar, A. Lehmusvuori, U. Karhunen M. Soikkeli, T. Seppä, E. Tuunainen, A. Spanger, P. Von Lode, K. Rantakokko-Jalava, G. Otto, S. Scheding, T. Soukka, S. Wittfooth and T. Laurell.

Poster presented Micronano System Workshop 2016, May 17-18, Lund, Sweden.

XI **Twenty second acoustofluidic whole blood hematocrit assay**

Klara Petersson, *Ola Jakobsson, Pelle Ohlsson, Per Augustsson and Thomas Laurell.*

Poster presented at μTAS 2016, October 9-13, Dublin, Ireland.

XII **Acoustofluidic whole blood hematocrit assay**

Klara Petersson, *Ola Jakobsson, Pelle Ohlsson, Per Augustsson and Thomas Laurell.*

Oral presentation at Acoustofluidics 2017, August 28-29, San Diego, California, USA.

Abbreviations

CNA	Circulating nucleic acid
CRP	C-reactive protein
DEP	Dielectrophoresis
DLD	Deterministic lateral displacement
ELISA	Enzyme-linked immunosorbent assay
EOF	Electroosmotic flow
HCT	Haematocrit
LOC	Lab-On-a-Chip
MS	Mass spectrometry
PCR	Polymerase chain reaction
PCV	Packed cell volume
PDMS	Polydimethylsiloxane
POC	Point of care
PSA	Prostate specific antigen
Re	Reynolds number
RBC	Red blood cell
SAW	Surface acoustic wave
TSH	Thyroid-stimulating hormone
WBC	White blood cell

Contents

1. Introduction

The miniaturisation of electronic devices and later the development of microelectromechanical systems (MEMS), demonstrated the possibilities to manufacture microscale structures in silicon for precise control of small volumes of liquid, starting the field of microfluidics. Liquid contained in micrometre channels behaves differently compared with the macroscopic world. Most prominent is the laminar flow enabling two liquids to flow side by side without mixing in other ways than diffusion compared with the more turbulent flow known on the macroscale in for example a river. As Manz *et al.* predicted in 1990[1], the field of microfluidics has grown big within life science applications. The expectations are to develop faster, cheaper and more sensitive assays to detect biomarkers from various samples, e.g. blood, saliva and urine.

Currently, most biomarker testing is performed by highly automated analysers in centralised lab facilities where samples are sent from hospitals and smaller clinics. This results in a delay of the answer because of transportation and administration. Some tests can be performed at the clinic or on the field by so called point-of-care devices (POC) and the sample to answer time can be considerably reduced. Lots of research in the microfluidics field focus on the development of these POC instruments and the possibilities to integrate several steps into a so called Lab-on-a-chip system (LOC), where sample preparation and detection can be performed in one single automated platform.

This thesis focuses on how a specific type of microfluidics, namely acoustophoresis or acoustofluidics, can contribute to facilitate blood based diagnostics. Blood samples are one of the most important sources of information in healthcare when mapping a patient's health status. Detection of biomarkers found in blood can be used for both diagnostic purposes and for following the response of a treatment. Almost half of a blood sample consist of blood cells while the other half, the blood plasma, contains many biomarkers. These biomarkers are often present in low concentrations and initial removal of the blood cells is often crucial for biomarker detection. Among other microfluidic methods, acoustophoresis in well suitable for microfluidic sample preparation and blood cell removal as well as integration into LOC systems.

Acoustophoresis, which means migration with sound, uses ultrasound in combination with microfluidic channels or cavities to move particles or cells in a controlled way. As ultrasound has wavelengths matching microfluidic channel dimensions, a standing wave can be created between the wall of a microfluidic channel. When introducing blood to an acoustofluidic device, blood cells can be moved and separated from the plasma which can be further investigated for biomarker recognitions.

In the forthcoming chapters, blood based diagnosis will be discussed as well as how microfluidics and specifically acoustofluidics can be used to facilitate sample preparation. Possibilities to integrate microfluidics and acoustofluidics to LOC devices in the development of POC devices will also be covered.

2. Blood based diagnosis

Blood is a gold mine of information useful in clinical care for diagnostic purposes, following disease progression, monitoring response to treatment or to determining a patient's prognosis. Information about the function of organs and tissue in the whole body is present in blood samples as blood is recirculated every minute in the body to transport for instance oxygen, nutrients, hormones and waste products. In this chapter, some basics about blood and its components will be covered as well as biomarkers found in blood and how they can be identified and quantified.

2.1 Blood components

Human blood consists of around 45 volume percent blood cells suspended in plasma, Figure 2:1. All of the blood cells are derived from the same hematopoietic stem cells found in the bone marrow. As the stem cell matures, they will differentiate into red blood cells, white blood cells and platelets.

Red blood cells

The most abundant of all human blood cells are the red blood cells (RBCs), also called erythrocytes (Table 2:1). The RBC content in a blood sample is often reported in volume percent, called the haematocrit (HCT). RBCs do not have a nucleus and are

shaped as a biconcave disk, giving them a large surface for gas exchange and high deformability capacity in order to be able to be squeezed through capillaries. The main function of the RBCs is the transportation of oxygen with the help of the protein haemoglobin. Haemoglobin is found in high concentration in RBCs and gives the blood its red colour. The cell membrane is covered by polysaccharides which forms antigens and gives the blood its blood type.[2]

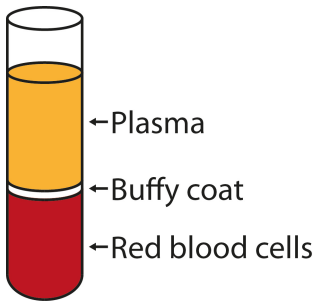


Figure 2:1. A blood sample divided by centrifugation into red blood cells, buffy coat and plasma. The buffy coat consists of platelets and white blood cells.

White blood cells

White blood cells (WBCs), also called leucocytes, are a collection of different cells that constitutes part of the immune system and are responsible for the protection of the body against foreign substances and pathogens. The number of WBCs in the blood is much lower than the number of RBCs, only 1 out of 1000 of all the blood cells is a WBC (Table 2:1). WBCs can be categorised in five subclasses with different tasks as neutrophils, eosinophils, basophils, monocytes and lymphocytes. In contrast to RBCs, all types of WBCs contain nuclei.[2]

Platelets

Platelets or thrombocytes, are small nuclear-free, discoid shaped cell fragments that are responsible for the blood clotting. The surface of a platelet is covered with proteins important for adhesion and aggregation, which in case of activation helps to form a platelet plug covering injures in the blood vessel. Their capacity to cross-link with the help of the plasma protein fibrinogen is one important step, among other, in blood clotting process.[2]

Plasma

Plasma is the straw-coloured liquid holding all the blood cells. In addition to water, plasma mainly consist of proteins. The most common proteins found in plasma are albumins, immunoglobulins and fibrinogen, all produced by the liver and lymphocytes. Different proteins have different tasks, of which one important role is to maintain the osmotic pressure and thereby regulate the blood volume. Some proteins are important for transportation of molecules that are not water soluble, e.g. hormones, vitamins and fatty acids. Another important protein group are the antibodies, which are used by the immune system in protection against foreign substances and organisms. Several proteins are involved in the important blood clotting process, which prevents bleeding. Plasma where all the clotting factors, such as fibrinogen, are removed is called serum. Except proteins, plasma also contains electrolytes, nutrients, hormones, waste products, RNA and DNA from cells and dissolved gases.[2]

Table 2:1: Normal cell count, size, shape, density and compressibility for different blood cells[2]

	Numbers	Size (µm)	Shape	Density (kg/m^3)	Compressibility (TPa^{-1})
Red cells	3.9-5.6x10^{12}/L (females) 4.5-6.5x10^{12}/L (males)	6.2 -8.2	Biconcave disk	1101[3]*	334[3]*
White cells (total)	4.0-11.0x10^9/L			1054[3]*	393[3]*
Neutrophils	2.5-7.5x10^9/L	10-12	Spherical	1075–1085[4]	
Lymphocytes	1.5-3.5x10^9/L	6-14	Spherical	1055–1070[4]	
Monocytes	0.2-0.8x10^9/L	12-20	Spherical	1055–1070[4]	
Eosinophils	0.04-0.44x10^9/L	10-12	Spherical	1075–1085[4]	
Basophils	0.01-0.1x10^9/L	8-10	Spherical	1075–1085[4]	
Platelets	150-400 x10^9/L	3x0.5	Discoid	1040-1060[4]	

*Mean value

2.2 Biomarkers in blood

A biomarker is a measurable substance that reflects a biological condition. Biomarkers are widely used for diagnostics and for following the treatment of a disease, and the number of available biomarkers has increased during the last decades mainly as a result of improved analytical methods.[5,6]. Biomarkers are not just present in blood samples; urine and saliva are also great sources of biomarkers. The following section introduce different types of biomarkers that can be found in blood samples.

Proteins

As previously mentioned, blood plasma is rich of with proteins and the complexity of the plasma proteome is huge. Not only can proteins functioning in plasma be found in blood but also messenger proteins on their way between tissues, proteins leaking out of tissue because of tissue damage or proteins released into the blood from tumours or foreign pathogens such as bacteria. Each protein can often be found in different glycosylated forms and in different sizes depending on the degree of maturity.[6]

Analysis of a single protein associated with a disease is the most common strategy today, but mass spectrometry-based quantitative proteomic technologies holds great promise in clinical care to get a protein map of a patient which not only can be used for diagnosis but also to reveal the state of a disease and the most appropriate treatment. This is often referred to as personalised medicine, where a patient's protein map can predict what treatment would be the most effective and what the chances for successful treatment are for the individual patient[7]. A large number of proteins associated with cancer have been identified[8] but the use of protein maps for classification of tumours among cancer patients is still mainly done at a research level due to the complexity of the plasma proteome and the variability depending on gender, age, the methods used for studying the proteins and the handling of the samples[5]. Still, there are single proteins associated with cancer that are used for diagnostic purposes. One of the most common biomarker for cancer is prostate-specific antigen (PSA) which is elevated in case of prostate cancer. The specificity of PSA is however low as other prostate related problems also results in elevated PSA levels.

C-reactive protein (CRP) is an unspecific marker for inflammation that has been known since 1930 but is still used in healthcare[9]. CRP is not suitable for diagnosis because of its unspecific nature but is frequently used to follow antibiotic treatment or more specifically the failure of antibiotic treatment but also the course of a disease in general[10]. CRP has also been used as a marker for cardiovascular risk[11]. Also hormones can be used as biomarkers, for instance elevated levels of the thyroid-stimulating hormone (TSH) indicate deficient production of thyroid hormones.

Bacteria

The blood stream is a sterile environment in a healthy person, but bacteria can enter the blood stream in case of an infection somewhere in the body, during surgery, during dental treatment, during tooth brushing or whenever a non-sterile object penetrates the skin. This bacterial infection is called bacteraemia and is usually something that the immune system can handle. Bacteraemia, as well as other infections, can lead to a severe immune response called sepsis which is a life-threating condition that requires immediate antibiotics treatment. Initial treatment is often done with broad-spectrum antibiotics but when the type of bacteria is identified the treatment can be directed to antibiotics targeting that specific bacteria.

Electrolytes

Just as for the proteins, changes in electrolyte concentration can indicate a disease state. Variations in electrolyte concentrations can be related to side effects from drugs, shortage of vitamins, kidney problems and liver problems. By measuring electrolyte concentration, you also get information about hydration, acid-base balance and ion balance.

Metabolites

Metabolites are molecules, including vitamins, fats, amino-acids, sugars and hormones, that are products of the metabolism. Some metabolite blood tests, such as glucose testing, have long been used in medicine but similar to protein biomarkers the recent improvements in analytical technology have increased both the interest for metabolites as biomarkers and the possibility to identify new markers[12]. Some of the metabolites, e.g. glucose, can be found in relatively high concentrations and can often be detected from small volume blood samples, such as finger-prick, and directly in whole blood even if studies have shown that plasma levels are more reliable than whole blood levels[13]. Blood contains endogenous metabolites, originating from tissues and cells within the body while metabolites which are not naturally produced in the body, e.g. drugs generally is studied in urine even if they also can be found in the blood.[14]

Cells

Abnormal numbers of different blood cells can also be used as biomarkers, and blood cell count is one of the most common blood tests performed. Changes in RBC count can indicate bleeding or doping and an increased WBC concentration can a be sign on infection. Cell morphology is another thing that can be of interest as it is associated to specific diseases, e.g. sickle cell anaemia can be recognised by the sickle shape of the RBCs. Circulating tumour cells, CTCs, are cancer cells that have detached from a tumour and entered the blood stream. They are of interest as the detection of them may add information about the risk of spread of the cancer.

Circulating nucleic acids

Circulating nucleic acids, CNAs, are traces of free DNA, mRNA or microRNA that can be found in blood sample among other body fluids. Even though their existence has been known since the 1950s, their potential as biomarkers in diagnostics has only recently been studied in more detail. CNAs have mostly been used for non-invasive prenatal diagnosis, as cancer marker or as a marker for transplant rejection[15]. Tumour detection and cancer progression can be followed in a non-invasive way as CNA concentration correlates with malignant progression in cancer[16-18]. Besides cancer, CNAs have shown to be associated with for example stroke severity[19] and malaria infection[20] and the recent improvements in genomic and molecular methods to investigate CNAs are expanding their range of applications[18].

Viruses

Viruses, are small nucleic acid-carrying protein encapsulated particles ranging from 20-400 nm, that can be found in the blood stream during viral infection. Human immunodeficiency virus (HIV) is identified through detection of specific antibodies or antigens but can also be detected by identification of virus RNA[21]. Most viruses found in the blood stream, like HIV, Dengue fever and Flu, are present in very low concentrations, a feature that makes them difficult to analyse.

Exosomes and microvesicles

Exosomes (30-100 nm) and microvesicles (50-1000 nm) are extracellular vesicles which can be found in blood and urine. These vesicles are released by cells because of stress, activation or apoptosis, and contain tissue signature, proteins and RNA. An advantage over protein biomarkers found directly in blood plasma is that the vesicles have a membrane structures protecting their content from proteases is the blood making them more stable over time.[22] Increased levels of extracellular vesicles have as an example been linked to cardiovascular disease[23,24]

2.3 Diagnostic methods

Huge amounts of blood samples are drawn and analysed every day and the results can help physicians to follow the response to treatment or to make a diagnosis. Some analyses are performed bedside, with the result available within minutes. Other samples are sent to centralised hospital labs, and the turnaround time can be several days. The number of diagnostic tools for different biomarkers is huge, and this section will not cover them all but rather focus on some methods for biomarkers relevant for this thesis.

Studying and counting cells

Cell counting has gone from historically being done manually by microscopy to being done by highly automated whole blood analysers. The cell counting is commonly based on the Coulter principle[25] where the change in impedance is measured when a diluted sample of cells pass through a narrow opening blocking an electrical current, Figure 2:2. The size of the impedance peak is used to calculate the cell volume. To obtain WBCs and platelet count, the RBCs are selectively lysed in the analyser before passing by a second detector where platelets and WBCs can be distinguished based on size. As the different WBCs are overlapping in size, they cannot be distinguished by the Coulter principle why optical methods may be preferred. In flow cytometry a dilute cell sample, ensuring that one cell at the time, passes through a flow cell and past a focused laser beam[26], Figure 2:2. By studying the scattering of light from the cells, which depends not only on the size but also the granularity of the cell, different cell types similar in

size can be distinguished. WBC types can also be distinguished from each other in flow cytometry by labelling with antibodies immobilised with different fluorophores. For RBCs, the Coulter principle based analyser calculates both the cell count and the mean cell volume which when multiplied gives the volume percent of red blood cells, called haematocrit (HCT). Normal values differ slightly between men (42-52%) and women (35-47%). Abnormal values can indicate several disease states and HCT is one of the most common blood analyses made. An alternative way to measure HCT is by centrifugation of a blood filled capillary[27]. The relative height of the packed blood cell to the total blood cell and plasma height is called packed cell volume (PVC). This can be done by haematocrit centrifuges placed in e.g. primary care units, whilst the whole blood analysers are placed in centralised lab facilities.

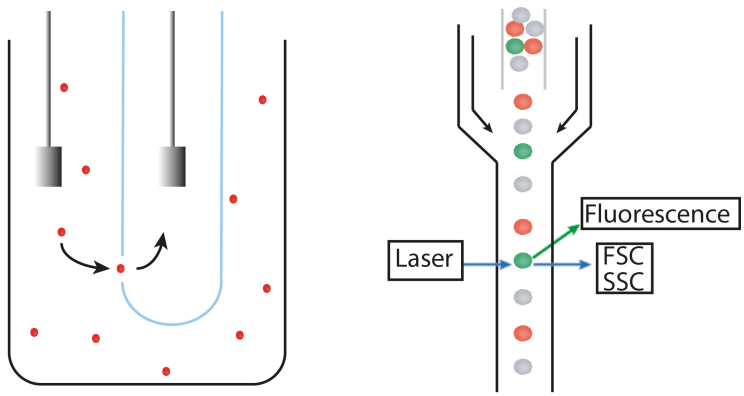


Figure 2:2: Left: Coulter counter, two chambers containing electrolyte and diluted sample. A change in electrical impedance is detected when sample is drawn through an opening, blocking the current, from one chamber to the other. The impedance change is proportional to the volume of the particle. Right: Flow cytometry, a diluted sample passes through a focused laser beam and forward scattering, side scattering and fluorescence are detected.

Erythrocyte sedimentation rate (ESR) is a general inflammation marker which, in the standard Westergren method, is measured by letting the blood sediment in a specific tube for one hour and then measure the high (in mm) of the plasma pillar over the sediment of RBCs. The reason for increased ESR in the presence of inflammation is that proteins (e.g. fibrinogen and immunoglobulins) in plasma make the red blood cells cluster in rolls by stacking the flat side to each other like a stack of coins, Figure 2:3. This formation of so called rouleaux leads to increased sedimentation rate because

of their increased particle size. Nowdays, ESR has been replaced by other inflammation markers, but is still used in some special cases. Even if the definition of ESR is the sedimentation length in one hour, there are automatic analysers that can perform the test faster. Some are based on the same principle, but with earlier readout extrapolated to the one hour value. Other faster methods are instead looking at how fast the rouleaux reappear after having exposed the blood to high shear forces to break up any rouleaux.

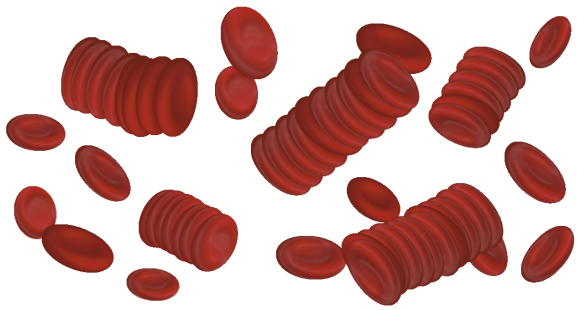


Figure 2:3: Red blood cells in rouleaux formation.

Bacteria detection

The gold standard for bacterial detection in blood is blood culture. A blood sample, typically 20-30 mL, is divided to different culturing bottles containing different growth media optimised for aerobic bacteria, anaerobic bacteria or fungal growth. The bottles are placed in 37°C culture chambers and CO_2 is monitored to detect microbial growth. Detection can take from hours to a few days up to two weeks. If no growth is detected within two weeks, the sample is considered negative.

Growth detection is automatized but the subsequent investigation to identify strain and antibiotic resistance is often done manually. A first step is to distinguish gram positive and gram negative bacteria by gram staining, which differentiates the bacteria based on properties of theirs cell walls. This information helps in selecting antibiotics for the subsequent antibiotic susceptibility test. Small wafers containing antibiotics are placed on agar plates with growing bacteria. If the antibiotic is effective, a clear spot will appear around the wafer as the bacteria won't grow in proximity of the antibiotic. Minimum inhibitory concentration, MIC, is then calculated to get the lowest concentration of antibiotic which prevents bacteria growth.

Polymerase chain reaction (PCR) and mass spectrometry (MS) may be used after the blood culturing to identify the bacteria species. The sensitivity of the blood culture limits the sensitivity of further investigations. Slow growing bacteria or blood samples taken after antibiotic treatment may not show growth even if bacteria are present[28]. There are PCR methods, e.g. SeptiFast (Roche Molecular Systems), where blood cells

are lysed and DNA extracted directly from whole blood without culturing. The drawback with these systems is that they often are labour intensive with many manual steps, which expose the sample to the risk of contamination.

Protein detection

Several methods have been developed for protein detection. Historically, in the 1970s, 2-D gel electrophoresis speeded up the field as several proteins could be studied at the same time[29,30]. Gel electrophoresis has also been used in combination with antibody recognition in so called Western blot to detect specific proteins. The next big breakthrough was when MS further accelerated the proteomics field, and still this is the main technique for studying proteins in research and biomarker discovery[7]. However, data from FDA (U.S. Food and Drug Administration) shows that around 80% of all protein biomarkers approved by them are identified by an immunoassay in a hospital laboratory[11].

The most frequently used immunoassay for clinical protein detection is the enzyme-linked immunosorbent assay (ELISA)[31]. In the 1960s, radioactive reporter labels linked to the antibodies where used in immunoassays (RIA), but there was an obvious safety concern with the radioactive compunds[32]. In 1971, a first paper on ELISA was published where an enzyme was used instead of a radioactive label[33]. There are different formats for immunoassays and ELISAs but common for all is the involvement of one or more antibodies which produce a measurable signal. In an ELISA, the enzyme linked to an antibody can convert a substrate to a measurable colour change. Despite the enzymes used in ELISA and the radioactive isotopes used in RIA, immunoassays can use detection antibodies labelled with fluorescent markers, Figure 2:3.

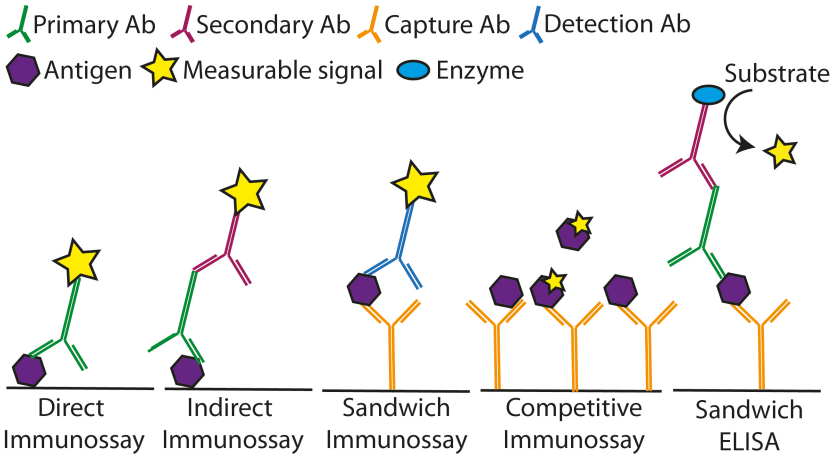


Figure 2:3: Direct immunoassay: The antigen binds to a surface and a labelled primary antibody binds to the antigen. Indirect immunoassay: The antigen binds to a surface and a primary antibody binds to the antigen followed by a labelled secondary antibody which binds the primary antibody. Sandwich immunoassay: A capturing antibody bound to a surface binds the antigen followed by a binding of labelled detection antibody to the antigen. Competitive immunoassay: Unlabelled antigen competes with labelled antigen to bind a capturing antibody. The more unlabelled antigen in the sample, the more labelled antigen will get displaced and the amount of unbound labelled antigen can be measured. Sandwich ELISA: A capturing antibody bound to a surface binds antigen and a primary antibody bind the antigen in a sandwich format. A secondary enzyme labelled antibody binds the primary antibody and when substrate is added the enzyme converts the substrate to a measurable signal.

3. Microfluidics

In the multidisciplinary field of lab-on-a-chip, the great advantage lies in the fluid and molecular behaviour on the microscale. When fluid flows are scaled down the surface to volume ratio increases and other phenomena become important than in the macro world. This is because the viscosity and surface forces starts to dominate over the inertia that is often negligible at this scale. Focus in this chapter will be on the effects important for the acoustofluidic applications presented in this thesis.

3.1 Laminar flow

One big difference between macrofluidics and microfluidics is the lack of turbulent flow in the latter. As flow lines constantly are mixed in the turbulent flow of a river going down a mountain, they are kept parallel without mixing in the microscopic world. If no extern force is applied, molecular exchange across the flow lines is governed by diffusion.

Motion of an object can be described by Newton's second law ($F=m^*a$, where F is the force on an object with the mass, m, and acceleration, a), based on motion of centre of mass. In the case of fluids, it is better described in terms of continuous fields as in the Navier-Stokes equation as the pressure, p, and a velocity, v, of a fluid with density ρ, dynamic viscosity η:

$$\rho\left[\frac{\partial v}{\partial t}+(v\times\nabla)v\right]=-\nabla p+\eta\nabla^2 v+f$$

where f is the body force and t is the time. For Re <<1 is this simplified to:

$$\rho\frac{\partial v}{\partial t}=-\nabla p+\eta\nabla^2 v+f$$

The reason for laminar flow is that viscous forces dominate over inertial forces. To predict laminar flow, the Reynolds number, Re, can be calculated from the Navier-Stokes equation by the ratio between inertial and viscous parameters:

$$Re=\frac{\rho L v}{\eta}$$

where L is the characteristic length. For low Re, <1500, viscosity dominates which results in laminar flow. As a result, you can predict and keep very precise and repeatable control of fluid flows.[34]

3.2 Flow profile

When a pressure-driven flow of a fluid flows through microfluidic channels there will be a viscous drag against the channel wall. This no-slip boundary condition leads to a parabolic flow profile with zero velocity at the wall that increases to a maximum in the centre (Poiseuille flow), Figure 3:1. This means that particles flowing in a microfluidic channel will move slower if they are closer to the side walls, the top or the bottom.

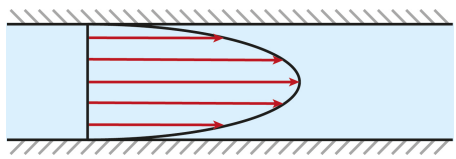


Figure 3:1: Velocity profile for pressure driven flow in a microfluidic channel. Zero flow at the walls and maximum flow in the centre.

3.3 Stokes drag

If a particle is moved by an external force, for example an acoustic force, the particle will experience a frictional force or drag force, Stokes' drag (F_{drag}). The particle will accelerate to a velocity, u, where the drag force equals the external force. F_{drag} is directed

in the opposite direction to the external force and can be calculated with the following formula if Re <1.

$$F_{drag} = 6\pi\eta a u$$

where η is the dynamic viscosity, a is the particle radius and u is the velocity of the particle. As seen in the equation above, Stokes drag scales with the radius of the particle, while many forces causing movement, such as gravity or the primary acoustic radiation force, scales with the volume. This means that Stokes drag becomes more dominant when the size of a particle is reduced.

3.4 Capillary action

In the interface between liquid and air, surface tension will appear as a result of the cohesive forces between liquid molecules. As the molecules at the surface do not have molecules all around them they cohere more strongly with each other than with the surrounding. The surface tension is for example responsible for the spherical shape of droplets.

When adhesive forces between a liquid and a surface pull the liquid meniscus up through a small channel, the surface tension will act to keep the liquid gas interface intact at certain angle, resulting in that the whole interface follows. This combination of the adhesive forces between surface and liquid and the surface tension is called capillary action and the force moving the liquid is called capillary force. As a result, flow can appear in small capillaries without any external force and sometimes opposite an external force (e.g. gravity). If the channel is sufficiently small the surface tension and forces between the liquid and channel wall will act so that the liquid is drawn into the channel, Figure 3:2.

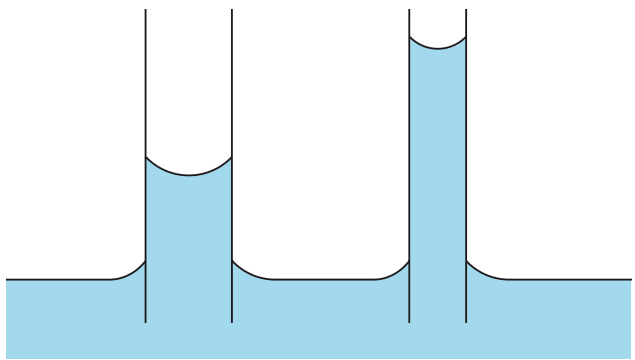


Figure 3:2: Liquid traveling up capillaries be the means of capillary action.

3.5 Diffusion at the microscale

In a microfluidic system with laminar flow the only contribution to mixing will be diffusion. Diffusion is the transport of molecules from high concentration to low concentration by random molecular motion. Even if diffusion is a very slow process, it is of high importance when working with microfluidics and small volumes and distances. Laminated liquids with low flow rates in a long channel with a small cross-sectional area will to some extent be mixed by diffusion.

4. Acoustofluidics

Acoustofluidics is a combination of microfluidics and the use of ultrasound to move particles or cells in microfluidic channels. In this way, particles can be separated, concentrated or washed in different manners. This movement of particles or cells by means of acoustic forces is also referred to as acoustophoresis. The movement of particles due to sound waves is not a newly realised phenomena. In 1874 Kundt showed that cork dust in a pipe, with a reflector at one end and instrument in the other, moved to the vibration nodes of a standing wave.[35]

4.1 Ultrasonic standing waves

Sound is a vibrations traveling through a medium such as air, a liquid or a solid. The human ear can register sound with frequencies between approximately 20 Hz and 20 kHz, frequencies above that span are called ultrasound and frequencies below infrasound. When two waves with the same frequency and amplitude travels towards each other a standing wave will form. This standing wave does not propagate but the amplitude of the standing wave in one point will vary with time. The location of the pressure amplitude minima is called a pressure node and the pressure amplitude maxima is called a pressure anti-node, Figure 4:1. A standing wave can be formed if two sound sources face each other and two waves with the same frequency meet.

Another way to form a standing wave is to let a propagating wave hit a reflector. The interference of the reflected wave and the original wave will then form a standing wave. Ultrasonic standing waves are standing waves where the sound waves are in the ultrasound regime. The wavelengths (λ) of these waves are of interest in microfluidics as they are in the same order of magnitude as the microfluidic structures.

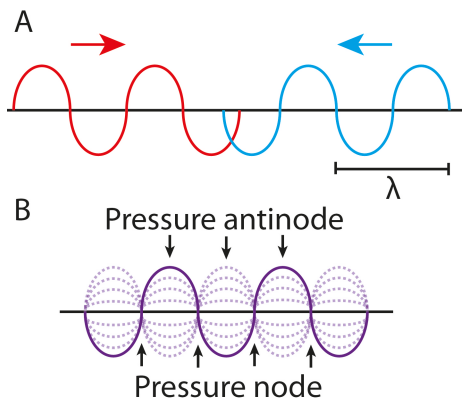


Figure 4:1: A: Two soundwaves with the same frequency and amplitude traveling toward each other. λ cis the wavelength. B: A standing wave oscillating between the two end positions. Pressure nodes are located where the amplitude is zero and pressure antinodes at maximum amplitude.

4.2 Primary radiation force

Some of the most important steps in the understanding of acoustic forces on particles in a suspension have been taken by King[36], Yosioka and Kawasima[37] and Gorkov[38] and they were recently summarised by Bruus[39]. Particles exposed to an ultrasonic standing wave field will experience a primary radiation force (F_{rad}) making them migrate towards pressure nodes or antinodes depending on their properties relative the suspending fluid. For a spherical particle with a radius much smaller than the wavelength, the primary radiation force, in a simplified form for a one dimensional standing wave, can be described as:

$$F_{rad} = 4\pi a^3 \Phi k_y E_{ac} \sin\left(2k_y y\right)$$

$$\Phi = \frac{\kappa_o - \kappa_p}{3\kappa_o} + \frac{\rho_p - \rho_0}{2\rho_p + \rho_o}$$

where a is the radius of the particle, Φ is the acoustic contrast factor, E_{ac} is the acoustic energy density, k_y is the wavenumber, y is the distance from the wall, κ_p is the compressibility of the particle, κ_o is the compressibility of the surrounding fluid, ρ_p is the particle density, and ρ_o is the density of the surrounding fluid. It should be noted that F_{rad} is highly dependent of the size of the particle. By balancing the primary radiation force and Stokes drag, the velocity (u_{rad}) of a particle relative the medium can be described as:

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

where η is the dynamic viscosity. The velocity of the particle is also dependent of the particle size, but not to the same extent as for the primary radiation force.

The primary radiation force also scales with the frequency and higher frequencies results in stronger force making acoustofluidics suitable for microfluidic systems. For particles or cells with positive contrast factor, the primary radiation force acts in the direction towards pressure nodes. Particles or cells with negative contrast factor will instead be pushed towards the pressure antinodes by the primary radiation force, Figure 4:2. The contrast factor is dependent on the density and compressibility of the particle or cell in comparison to the surrounding fluid. For most cells or bacteria present in a buffer, the contrast factor is more or less always positive, meaning that they will move towards the pressure node unlike fat particles, which is less dens than the medium, and would move towards the pressure antinode.

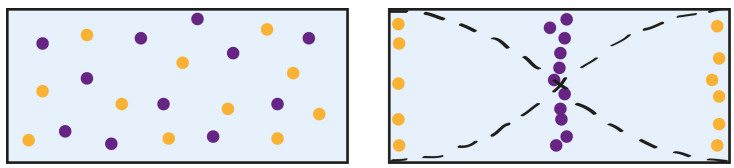


Figure 4:2: Left: Purple and yellow particles distributed in the cross section of a microchannel. Right: A standing wave is actuated between the walls of the microchannel. Purple particles with positive contrast factor move to the pressure node located in the middle. Yellow particles with negative contrast factor move to the pressure antinodes located close to the walls.

4.3 Secondary radiation force

When particles are coming close to each other, they will also experience a secondary radiation force (or Bjerknes force [40]) coming from the scattering of sound between particles. There are two terms responsible for the secondary radiation force, one that always is attractive and one that depends on angle, Figure 4:3. This results in an

equilibrium distance between particles experiencing this force which depends on the angle. Sometimes the optimal distance is zero bringing two particles in close contact with each other and sometimes they will be held at a small distance.

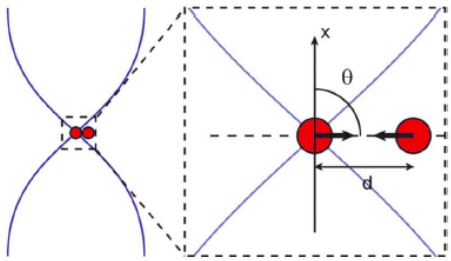


Figure 4:3: The secondary acoustic force is dependent on the angle θ, and the distance d, between the particles. Reprinted with permission from reference [41] Copyright (2012) Royal Society of Chemistry.

This force, which in most cases is much weaker than the primary force, is highly dependent of the distance between the particles and thereby only relevant when working with very high concentrations or acoustic seed trapping, which will be further described in section 4.5. The secondary radiation force (F_{sec}) between two particles with the same size can be described as:

$$F_{sec} = 4\pi a^6 \left(\frac{\left(\rho_p - \rho_o\right)^2 (3cos^2\theta - 1)}{6\rho_o d^4} v^2(x) - \frac{\omega^2 \rho_o \left(\kappa_p - \kappa_o\right)^2}{9d^2} p^2(x) \right)$$

where v is the velocity field, p is the pressure field, ω is the angular frequency, d is the distance between particles, and θ is the angle between the axis of the incident wave and the centreline between the two particles.[42,43]

4.4 Acoustic streaming

The acoustic field also generates acoustic streaming in the fluid, disturbing the laminar flow. This streaming occurs due to the shear stress near the walls in the microfluidic channel[44]. When having a half wavelength standing wave in a microfluidic channel, as in Figure 4:4, four streaming rolls will appear and circulate the fluid. As a result of this, particles focused to the centre pressure node will be influenced by a drag force generated by the streaming rolls to the bottom and top of the channel but they will still be kept centred laterally by the primary radiation force. However, this transportation of

particles is slow and in an acoustofluidic device it looks more like the cross-section seen in Figure 4:2. The acoustic streaming was first described by Lord Rayleigh and is sometimes referred to as Rayleigh streaming[45]. The acoustic streaming has later also been described by others[46,47].

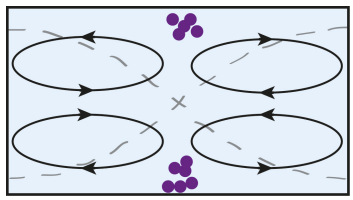


Figure 4:4: Rayleigh streaming in a microfluidic channel with a half wavelength standing wave. Purple particles are focused to the centre pressure node and the streaming moves them towards the bottom and top of channel.

For larger particles, the acoustic radiation force will dominate over the acoustic streaming, and they will be focused to pressure node. Smaller particles, with diameter < 2µm, will not experience a strong enough radiation force to dominate over the streaming. Instead of being focused to the pressure node, they will follow the streaming rolls. [48,49]

4.5 Acoustofluidic device

There are several types of acoustofluidic devices used to manipulate particles and cells on the microscale. The separation devices described in paper I-IV all utilised so called bulk acoustic waves. An acoustic trap was additionally used for the concentration of bacteria in the project described in paper II. Unlike the bulk waves used in this thesis, surface acoustic waves (SAW) have also been used for particle and cell manipulation. A short introduction to the different types of acoustofluidic devices will follow.

Bulk acoustic device

The acoustofluidic device is fabricated such that the dimensions of a channel or a cavity are matched to the wavelength such that and acoustic resonance is obtained at given actuation frequencies. This means that a pressure node or antinode can be placed in a preferable streamline within a microfluidic channel to move particles by the acoustic radiation force in a predictable path, Figure 4:5. Acoustofluidic devices are often made in silicon or glass because of their high contrast in acoustic impedance (density times speed of sound) and low acoustic damping, leading to good acoustic reflection and minimal loss of acoustic energy. Silicon channels with glass lids are practical as precise

structures can be made by etching, both materials have good reflecting properties and at the same time have visual access to the channel.[50] All separation devices presented in paper I-IV were made in silicon and/or glass and had a half wavelength matched to the width of the channel, resulting in a pressure node along the centreline and pressure antinodes close to the walls as in Figure 4:4.

The appropriate frequency, *f*, to use in these acoustofluidic systems can be calculated from

$$f = \frac{c}{\lambda}$$

where c is the speed of sound and λ is the wavelength. All acoustofluidic separation devices presented in paper I-IV had a width of approximately 375-400 µm being designed for a wavelength of 750-800 µm and with the speed of sound in water of 1484 m/s an optimal operating frequency of 1.85-1.97MHz.

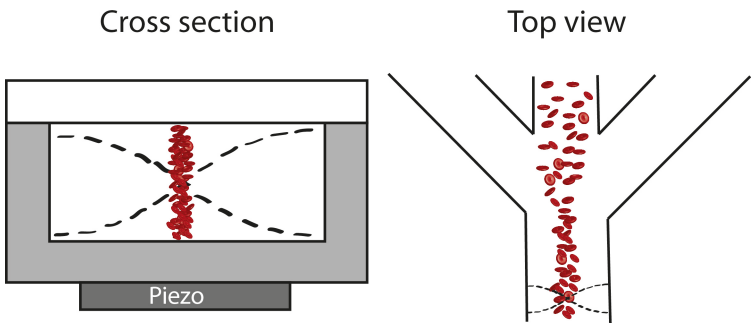


Figure 4:5: Left: Cross section view of a bulk acoustofluidic device with channel in silicon and a glass lid. Piezo transducer is glued underneath and the device is actuated with a half wavelength standing wave across the width. Right: Top view of the device with a standing wave between the side walls and a bifurcation at the end to enable separation.

To actuate the ultrasonic standing wave, a piezoelectric transducer is glued underneath the channel. The resulting direction of the wave, in width or height, depends on the best match between the dimensions and the wavelength. If a device for example have both height and width matching with the wavelength, there can be standing waves in both directions. This can be used to focus all particles to the very centre streamline, resulting in the same velocity for all particles compared with to a band where particles close to the walls (top and bottom Figure 4:2) will flow slower than particles close to the centre.[51] If particles are located in different streamlines (having different velocity due to the Poiseuille flow profile), they will spend different time in the ultrasonic standing wave field and their final lateral position will depend on their initial position. This especially becomes important when trying to separate

different particles or cell types to different streamlines, but is not as important for the work presented within this thesis where either all particles were ideally focused or the difference between the cell types was very large.

Acoustic trapping device

In the sepsis project described in paper II an acoustic trap was used for bacteria concentration. By attaching a small piezoelectric transducer to a glass capillary, a local sound field can be formed just above the transducer. The large gradient, which is formed when the sound field is strong over the transducer but not elsewhere, can be used to trap cells or particles, Figure 4:6. Seed trapping can be used if particles are too small to be trapped by the highly size dependent primary radiation force. Seed trapping starts with trapping of larger particles followed by trapping of smaller particles due to secondary radiation force when small particles come close to the bigger ones. In the sepsis project described in paper II 12 µm polystyrene beads were first trapped followed by trapping of bacteria. The trapped cluster can be released in a small volume in order to achieve concentration.

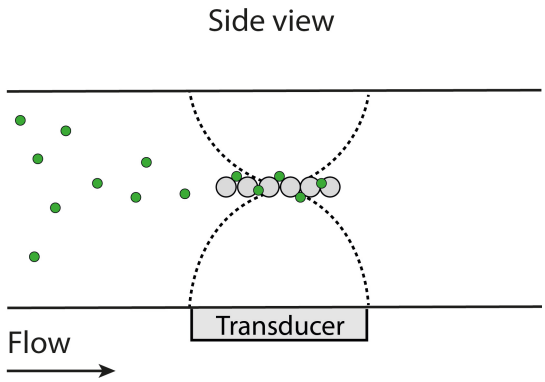


Figure 4:6: An acoustic trap can consist of a glass capillary with a small transducer attached underneath. The strong sound field over the transducer enables trapping in the standing wave between the top and bottom. Larger particles (grey) which are easy to trap act as seed particles and enables trapping of the smaller (green) particles due to the secondary radiation force.

Surface acoustic device

There is also another type of acoustofluidic separation device based on surface acoustic waves (SAW), traveling along the surface of a substrate, instead of the ones described above which are referred to as bulk acoustic waves. These surface waves are often generated by arrays of interdigital transducers deposited on to a piezoelectric material covered by a PDMS channel and do not rely on channels with good reflection properties. However, the standing wave acoustic energies (seldom reported) seem to be

much lower than the bulk waves, presumably due to the dissipative PDMS capping, why low flow rates and throughput is a common denominator in most reports on SAW based acoustofluidic separation.

5. Blood plasma separation

With some exceptions, e.g. POC glucose measurements, most biomarkers are not detected in whole blood but rather in the blood plasma as the blood cells often interfere with the accuracy of the detection method. Close to 70% of all errors in laboratory medicine can be derived from mistakes during sample collection and preparation[52] and in clinical studies sample preparation can heavily bias CV of the study results[14]. Standardised and automated methods are preferable to minimise variation and human error. Depending on biomarker stability, the time from sampling to blood plasma separation and detection can impact the results significantly. Also gentle removal of cells is favourable to avoid intracellular components to be released into the plasma. In this chapter, conventional methods for blood plasma separation as well as microfluidic and acoustofluidic methods will be discussed.

5.1 Conventional blood plasma separation

Depending on the blood volume to be processed, different macro-methods for blood plasma separation can be used. For blood transfusion, different blood components may be separated in a centrifugation based process called plasmapheresis. Plasmapheresis can be performed continuously when blood is drawn from a patient simultaneously as plasma is collected while blood cells are reinfused. There are also filter based methods,

like haemodialysis or cascade filtration, that can be used for large volume blood component separation. When generating plasma for diagnostic applications, which is the focus of this thesis, smaller blood samples in the mL range are used. The standard method for blood plasma separation in hospital clinical chemistry laboratories is centrifugation, but a few other methods can also be used.

Centrifugation

Centrifugation is a widely used method and the gold standard for blood plasma separation for diagnostic purposes. Blood samples are drawn into vacuum tubes containing anticoagulants and placed in a centrifuge. The tubes are then rotated at high speed and the blood cells are pushed towards the bottom of the tube (outward) by the centrifugal force. RBCs will be found in the bottom, WBCs and platelets will appear as the buffy coat in the middle and the clear plasma on top, Figure 2:1. Higher resolution in separation can be reached if the blood is centrifuged in a density medium. This minimises the contamination of the different types of blood cells in the different layers. Centrifugation is a well-established method that is reproducible, can simultaneously process large volumes and several samples and gives a high purity, but it is bulky and limited to batch-processing.

Other methods

Even if it is not often used in blood based diagnostics in hospitals, filtration is a method that can be used prior to analysis to remove blood cells. Blood filtration is usually done by a filter attached to a syringe. The obvious drawback with filtration is the small volumes that can be processed without clogging the filters and absorption of analyte to the filter membrane. Another way to get rid of the blood cells without separation is to lyse them. This can be done either chemically or mechanically and results in a plasma additionally containing cell debris and molecular content from the cells. This can be useful if the marker of interest is located inside blood cells. However, depending on the marker of interest, this may be a drawback as it releases intracellular content which can interfere with many detection methods. When for example looking for free DNA in plasma, lysis of white blood cells will increase the DNA background of the sample making the detection harder.

5.2 Microfluidic blood plasma separation

Microfluidics offers the possibilities of automated, portable or desktop devices, reducing user errors, process variations and sample to answer time. Because of the precise control of liquids and small distances, many assays can be performed in reduced time compared to conventional methods. The growing interest for the use of biomarkers for fast detection and for personalised medicine has increased the number

of available miniaturised biomarker detection methods recently. Some are aiming on completely portable systems that can be used in the field and in low resource areas while another approach may be an automated desktop device to use in the clinic as opposed to transporting samples to central labs. Even if the aim is automated systems that can handle the process from whole blood to answer in one integrated so called lab-on-a-chip or point-of-care device, many of the reported miniaturised detection methods still rely on initial blood plasma separation by conventional centrifugation. This absence of integrated microfluidic blood plasma separation has been described as the weak link in miniaturised detection systems and is a barrier for taking the lab-on-a-chip devices to the clinical market[53]. Several microfluidic blood plasma separation methods have been reported, even though they have rarely been integrated into lab-on-a-chip devices. When plasma validation is included, it is often done by biomarker detection in extracted plasma off chip but in a few cases the assay is completely integrated with blood plasma separation. Some microfluidic blood plasma separation methods are passive, based on channel geometry, cell behaviour, inertial effects and/or hydrodynamic forces. Active methods on the other hand rely on external forces such as acoustic, magnetic or electric forces.

Microfiltration

A straight forward and passive method for blood plasma separation is microfiltration where separation is performed based on size. Different types of filters made up of pillars, pores, membranes or packed beads can be used for the size exclusion of blood cells. Already in 1994, Wilding *et al.* presented a microfluidic filter inside a microfluidic channel fabricated in silicon and predicted it to be useful for analytical purposes.[54] With a more easy fabrication process several groups have incorporated commercially available blood separation membranes into microfluidics devices.[55-57] A simple filtration device can for instance be assembled by introducing a membrane between a polymer lid and a polymer bottom containing microfluidic channels. Plasma can move through the membrane by capillary force and further into the microfluidic channel while blood cells are physically restricted by the membrane. Small pores in the membrane are preferred to achieve a high blood cell removal efficiency, but this also results in small sample volumes that can be processed before clogging of the membrane. Diluted samples are often required to avoid clogging. To solve this Gong *et al.* scaled up the separation volume by using a large filtering area of 15 cm^2 which enabled separation of 1 mL undiluted whole blood.[57]

Filters can also be fabricated in the enclosed microfluidic channel either by a photo-polymerisation inside a microchannel[58] or by just simply letting packed microbeads act as filters.[59-61] Photo-polymerisation has the advantage that the filter can be placed where preferred without any constriction, which is needed when using packed microbeads. On the other hand, the packed bead filters are much easier to fabricate. The possible volume that can processed is limited as the packed bed eventually will be clogged, similarly to the membrane filter. Lee *et al.* showed another type of filter made in the

microfluidic channel during the sample run. A blood sample was drawn, by capillary force, into a plastic channel with hydrophobic patches where blood cells were captured while plasma continued through the cell-made filter.[62] The different microfiltration methods mentioned above are listed with their performance in Table 5:1.

Table 5:1: Blood plasma microfiltration devices

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Polypropylene membrane enclosed by PDMS [55]	-	-	20% HCT	300 µL	No haemolysis due to microscopy
Chromatographic paper, 2 µm, enclosed by PMMA (0.4 µm pores → to little plasma[56]	81.7% (99.9%)	-	8.5x dilution	40 µL	89.9 % HIV recovery detected off-chip
Polusulfone membrane enclosed by PMMA[57]	RBC: 99.9% WBC 96.9%	-	Undiluted	1 mL	Hepatitis b detection off-chip
Emulsion photo-polymerisation inside microchannel[58]	Similar to centrifuge	-	20x dilution(rabbit)	20 µL	No haemolysis or RBC leakage (off-chip G-6PHD assay, intracellular enzyme)
Mixture of beads in microfluidic channel[59,61]	-	1.9%	Undiluted (sheep)	10 µL in 600 s	Integrated IgG detection
Mixture of beads at microchannel inlet[60]	-	7%	Undiluted	5 µL in 110 s	
Hydrophobic patches capturing blood cells but not plasma[62]	-	3.4%	Undiluted	3 µL in10 min	

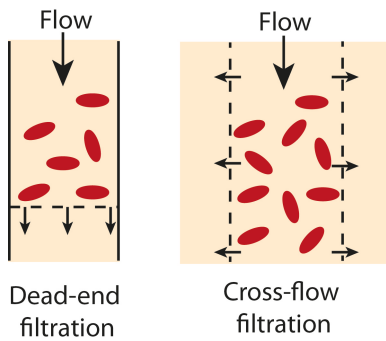


Figure 5:1: Illustration of the difference in dead-end filtration and cross-flow filtration. In dead-end filtration the flow is transverse to the filter, blood cells accumulate on the filter and will eventually clog it. In cross-flow filtration the filter is parallel to the flow, which slows down the clogging of the filter even if not avoiding it completely.

In spite of generating plasma usable for diagnostics, dead-end filtration often requires highly diluted samples or very small samples not to clog the filter as captured blood cells build up in front of the filter and eventually block it, Figure 5:1. To reduce clogging of the filters, the main flow in cross-flow filtration is parallel to the filter. In this way blood cells are constantly washed away and the volume that can be processed increases. One way to fabricate cross-flow filters is to enclose a commercially available membrane between two polymer pieces both containing microfluidic structures.[63,64] Branching separation channels from a main channel can also be fabricated directly in substrate.[65-67] Deformable RBCs can squeeze through constrictions smaller than their diameter, meaning that smaller pore sizes are needed which adds to the clogging problem. Even if cross-flow filtration is utilised, the total volume to be processed is small or diluted samples are needed to avoid clogging. Van Delinder *et al.* showed that they could reduce clogging by having a pulsating flow[66] and Aran *et al.* showed that highly heparinised blood samples or heparin treated membrane lessen blood cell adhesion to the membranes[63]. Axial migration, or the Fåhraeus effect, can also be utilised to minimize clogging in a cross-flow device.[68] This phenomenon will be further described under hydrodynamic separation. Since the yield of plasma is quite small, these methods are mostly suitable for detection of biomarkers present in high concentration to ensure that the sample contains enough markers. The performance characteristics of the devices mentioned above are listed in Table 5:2.

Table 5:2: Blood plasma cross-flow filtration devices

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Silicon glass device, centre blood channel with branches to two parallel plasma channels[65]	High	0.3-0.9%	2x dilution	5 µL in 110 s	No haemolysis due to microscopy
PDMS glass device, centre blood channel with branches to two parallel plasma channels[66]	-	8%	20% HCT	0.5 mL in 1 h	0.08% haemolysis Pulsating flow to minimise clogging
PDMS device with porous polycarbonate membrane, 200 nm pores[63]	100%*	15%	27-30% HCT	-	Detection of cytokines off-chip, 80% recovery. No haemolysis*
PMMA device with polycarbonate membrane, 1 µm pores[64]	RBC 93.9% WBC 82.9%	-	40% HCT	350 µL in 7.5 min	
PDMS device with branching channels[67]	98%	2%	Undiluted	5 µL in 3-5 min	Integrated detection of TSH
Glass device with porous membrane[68]	Cell free*	34%	Undiluted	200 µL 10 µL/min	Interfaced detection of CRP with ELISA

* Data not shown

Sedimentation assisted filtration

A way to minimize clogging of microfilters is to make use of sedimentation. On the macro-scale, blood cells will sediment over time. In a microfluidic structure, where the distances are small, blood cell sedimentation occurs in a more reasonable time frame. By placing a filter structure opposite the sedimentation direction either in the dead-end filtration way[69] or in a cross-flow manner[70], clogging can be reduced. Maria *et al.* showed a filtering by having a hydrophobic patch in a vertical channel. RBCs were trapped and sedimented while acting as filter for the plasma traveling upward by capillary force.[71] Sedimentation has also been shown to facilitate membrane filtration when the membrane was placed vertically in a chamber.[72] Another way to utilise sedimentation is to include weir-structures into the channel. It has been shown to work both with a single weir[73] or with weirs in series[74] where blood cells sediments.

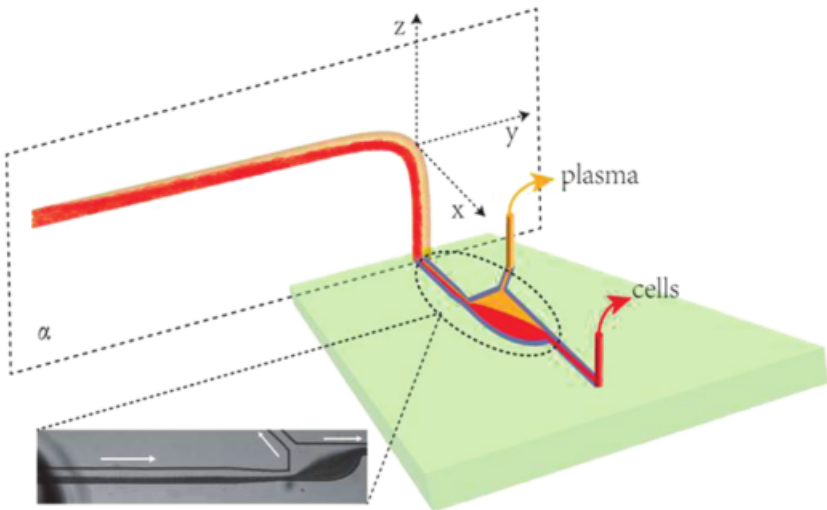


Figure 5:2: Slow flow in glass capillary increases sedimentation. Laminar flow was kept in a glass capillary connected to a glass chip where plasma was removed when entering a wider chamber. Reprinted with permission from reference [75]. Copyright (2012) American Chemical Society.

Sedimentation has also been utilised in a wider glass capillary which was connected to a microfluidic glass chip while laminar streamlines were retained and plasma could be extracted from the plasma streamlines, Figure 5:2[75]. Sedimentation can assist filtration by reducing the clogging problem. However, slow sample flows are desirable for sedimentation to have time to take place, making sedimentation, like most microfiltration methods, most suitable for small fingerpick blood samples for detection

of biomarkers present in high concentration. Performance of a few sedimentations based methods are presented in Table 5:3.

Table 5:3: Sedimentation assisted blood plasma separation devices

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Sediment assisted cross-flow filtration device[70]	-	7%	6x diluted by co-flow	3 µL 1 µL/min	<1 mg/dL haemoglobin
Weir structures in PDMS, streptavidin printed glass lid[73]	99-100%	-	Undiluted	5 µL in 2 min	Integrated streptavidin-biotin assay (10 min)
Weir structures in PDMS, glass lid, 7 parallel channels[74]	100%	-	0.2-20% HCT	37 µL	No haemolysis (shear stress calculations)
Sedimentation in glass capillary and extraction in glass chip (Figure 5:2)[75]	95-99%	66%	8% HCT	15-60 µL/min 4h	<1 mg/dL haemoglobin, theophylline detection off chip (no difference compared to centrifuge)
Large volume chamber with vertical asymmetric polysulfone membrane[72]	-	15%	Undiluted	1.8 mL in 7 min	Low hemolysis, Detection of HIV of chip with high recovery.
Vertical channel with membrane on top[69]	-	20%	10x diluted	12 µL in 20 min	90% of proteins and 100% of spiked cDNAs compared to centrifuge
Vertical channel with hydrophobic patch where blood cells get trapped and sediments[71]	99.9%	20%	Undiluted	10 µL	Integrated glucose strip (no difference compared to centrifuge)

Hydrodynamic separation

Hydrodynamic separation, based on hydrodynamic effects on the blood cells in straight channels and geometrical structures of the micro channels, has been used for continuous blood plasma separation. Deformable RBC migrates away from the channel wall towards centreline during flow in microchannels due to viscous lift forces when viscous forces dominates (Reynolds numbers < 1). This effect, called Fåhraeus effect[76], was first described in blood vessels where the average concentration of blood cells decreases as the diameter of the vessel decreases due to this migration in combination with Poiseuille flow, meaning higher flow rate in the centre than close to the vessel walls. The Fåhraeus-Lindqvist effect[77] is described as the viscosity decreases in blood when the diameter of the vessel decreases and is a result of the Fåhraeus effect. The Fåhraeus effect, also called margination since the RBCs displace the WBCs to the margins of the blood vessels or channels, has been used in microfluidic channels to create a cell free layer close to walls. Another phenomenon that has been widely used in blood plasma separation on the micro scale is the Zweifach-Fung effect[78], also called plasma skimming, stating that when blood flows by a bifurcation, blood cells have a tendency to travel into the channel (or vessel) with the highest flow rate.

The cell-free layer created by the Fåhraeus effect can be enhanced by letting the blood flow first through a constriction, resulting in high shear stress which pushes the cells even more to the centre, followed by an expansion, Figure 5:3, left.[79] In this way higher plasma volumes can be extracted without lowering blood cell removal. Browne *et al.* utilised the Fåhraeus effect for blood plasma separation in another way. Blood cells were focused to the channel centre where they experienced a higher flow rate, resulting in packed blood cells in the front of the blood flowing through a long channel.[80] By having channel structures close to cell dimensions, the Zweifach-Fung effect can be utilised for blood plasma separation when the flow ratio between the main channel and the branching channels is high enough.[81] However, small channel dimensions increase the risk for clogging which restricts throughput. Jaggi *et al.* solved this by high aspect ratio channels where one dimension still was kept small, Figure 5:3 right. This device could run undiluted whole blood at a flow rate of 5 ml/min but with a low blood cell removal. Initial dilution of blood increased the blood cell removal significantly.[82] Still, blood separation by Zweifach-Fung effect is most suitable for small blood volumes or diluted blood samples to avoid clogging and reach sufficient blood cell removal. Integration of biomarker detection has successfully been shown from just finger prick volumes of blood.[83] Separation performance of these methods are presented in Table 5:4.

Table 5:4: Hydrodynamic blood plasma separation.

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Fåhraeus effect + constriction-expansion (Figure 5:3)[79]	-	13%*	16% HCT washed cells	1 mL 200 µL/min	
Zweifach-Fung effect, 6:1 flow rate ratio[81]	100%	15-25%	10-36% HCT	10 µL/h	Defibrinated sheep blood. No haemolysis*
Zweifach-Fung effect, high aspect ratios to achieve high throughput (Figure 5:3)[82]	30% (92%)	4% (2.5%)	Undiluted (4.5% HCT)	5 mL/min	No haemolysis (shear rate calculations)
Zweifach-Fung effect, integrated barcode chip[83]	-	6%*	5% HCT*	Finger prick	Integrated detection of 8 cancer related proteins
Fåhraeus effect, blood cells collected in front of flow[80]	-	-	5-60% HCT	0.5-1.5 µL	Integrated ELISA for heart attack biomarker detection

* Calculated from whole blood = 45% HCT

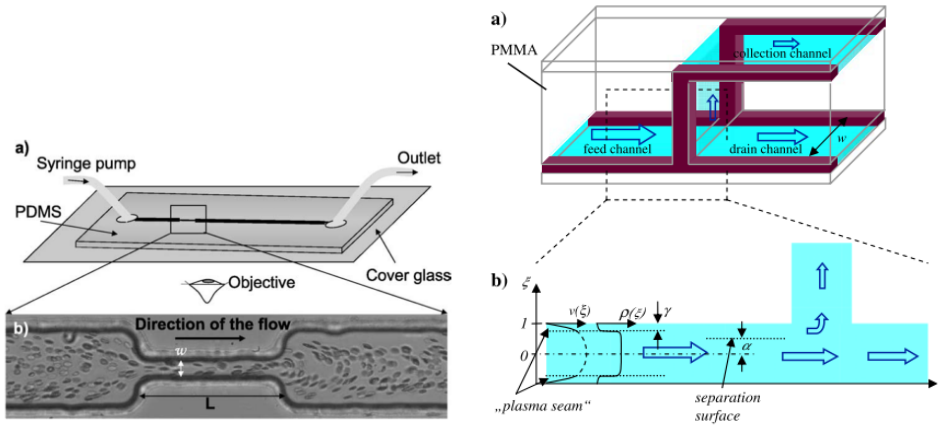


Figure 5:3: Left: Constriction followed by expansion to extend the cell free layer. Reprinted with permission from reference [79]. Copyright (2006) IOS Press., Right: High aspect ratio channels for high throughput separation based on Zweifach-Fung effect. Reprinted with permission from reference [82]. Copyright (2006) Springer Nature.

For high flow rates, Reynolds numbers increase and inertial forces start to dominate over viscous forces. As a result, deformable blood cells will move according to inertial forces instead of viscous forces (the Fåhraeus effect). There is also a regime, when the Reynolds number is close to 1, where both viscous and inertial forces contribute, leading to an even stronger lift force.[84] Blood plasma separation has been achieved by the Fåhraeus effect and constriction-expansion in combination with inertial lift force due to high flow rates.[85,86] High flow rates with a resulting inertial lift force in combination with the Fåhraeus effect can be used to facilitate a Zweifach-Fung device for blood plasma separation.[87,88]

Apart from the combinations of forces and channel geometries mentioned above, the temperature has also been shown to affect the extension of the cell free layer. A temperature increased from room temperature to 50 degrees Celsius was shown to increase the cell free layer by 250%.[89] However, these high temperatures are not practical when working with blood samples. Blatter *et al.* instead made use of centrifugal forces with bends introduced to the microfluidic channel to extend the cell free layer.[90,91] Centrifugal forces have also been used in combination with the Fåhraeus effect, constriction-expansion and Zweifach-Fung effect, to increase the cell free layer, Figure 5:4.[92-94] Common for blood plasma separation by hydrodynamic effects is that they are suitable for continuous blood plasma separation as clogging is less of a problem than for filtration methods. The throughput is often relatively high but undiluted blood in general give a very low plasma yield or a low blood cell removal. The separation performance of respective device can be seen in Table 5:5.

Table 5:5: Hydrodynamic blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Fåhraeus effect + constriction-expansion + inertial lift force[85]	99%	10.7%	20x diluted	100 µL/min	No haemolysis, total protein and aspartate alanine aminotransferase activity similar to centrifuge
Fåhraeus effect + constriction-expansion + Zweifach-Fung effect + inertial lift force[86]	99%	15-17%	20 x diluted	50-175 µL/min	No haemolysis, proteomic profile similar to centrifuge
Fåhraeus effect + constriction-expansion + Zweifach-Fung effect + inertial lift force[87]	53%	40%	3% HCT	10 mL/h	
Fåhraeus effect + constriction-expansion + Zweifach-Fung effect + inertial lift force[88]	100%	5%	Undiluted	2 mL/h	Detection of cDNA off chip
Fåhraeus effect + constriction-expansion + Zweifch-Fung effect + temp. 37° [89]	97%	3.5%	30% HCT	200 µL/min	250% wider cell-free layer at 50 °C
Fåhraeus effect + Zweifach-Fung effect+ centrifugal forces[91]	90%	5-10%	5% HCT	60-1200 µL/min	
Fåhraeus effect + constriction-expansion + Zweifach-Fung effect+ centrifugal forces (Figure 5:4)[94]	99.5%	1-6%	Undiluted	0.5 mL/min	Haemoglobin, glucose, hCG and total protein concentration similar to centrifuge

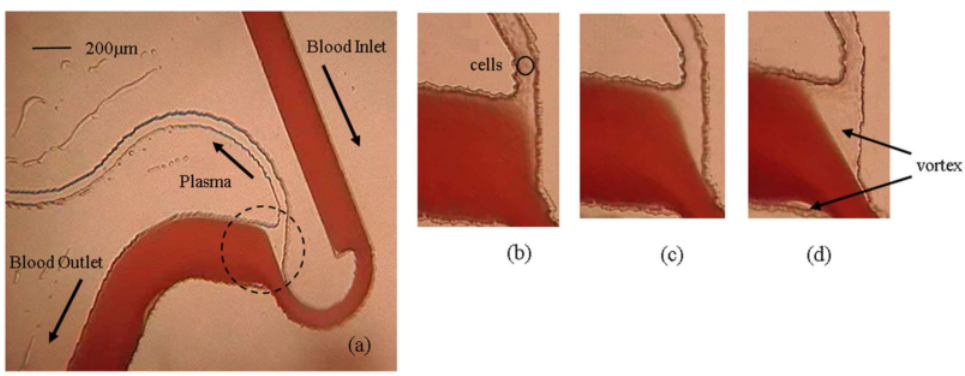


Figure 5:4: A combination of the Fåhraeus effect followed by constriction-expansion, centrifugal forces and Zweifach-Fung effect to separate plasma from blood. Reprinted with permission from reference [94]. Copyright (2016) Springer Nature.

Inertial separation

As mentioned above, inertia cannot be neglected when the Reynolds number approaches 1. Viscous and inertial forces can co-exist and laminar flow behaviour of microfluidics is observed for Reynolds numbers up to 2000. Cells can be focused to equilibrium position by balancing the inertial lift force from the wall and the shear gradient lift force that arise from the parabolic flow profile.[95] Blood cells are then focused to bands with plasma between them and by expanding the channel the centre plasma fraction can be increased.[96] By introducing a curvature in the channel geometry a secondary flow arises due to the mismatch in flow velocity between flow in the centre of channel and flow close to the wall. Fluid flowing along the centre line has larger inertia which results in a flow outward around a curve and as the channel is closed it will recirculate creating two vortexes. This flow, called Dean flow, has in microfluidics been used for mixing, but later also for particle separation as a balance between inertial lift force from the wall and Dean flow can position particles in the channel.[84,95] This balance between the inertial lift force and the Dean drag force leads to a faster migration to a more stable equilibrium position compared to inertial microfluidics in straight channels.[97] Initially experiments on doing centrifugation, not by spinning the chip but rather have a curved channel showed a promising channel structure for the use of Dean flow based separation which later has shown useful for blood plasma separation.[98] In a straight channel with constrictions followed by expansions the flow can experience Dean flow when entering a constriction and internal lift when flowing through, and in this manner balance the blood cells to an equilibrium position.[99] Inertial microfluidics is promising for high throughput separation because of their high flow rates but dilutions are necessary for the inertial forces to work, see performance in Table 5:6.

Table 5:6: Inertial blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Inertial focusing + expansion [96]	-	-	5% HCT	200 µL/min	80% bacteria recovery, parallelisation → 8 mL/min
Spiral channel, balance between inertial lift force and Dean flow[98]	100%	-	0.45% HCT	1 mL/min	
Constrictions-expansions, balance between inertial lift force and Dean flow[99]	60%	62,2%	11x diluted	13.2 mL/h	

Deterministic lateral displacement

Deterministic lateral displacement (DLD) is a hydrodynamic microfluidic separation method first introduced by Huang in 2004[100]. The technology is based on arrays of pillars within a microfluidics channel where each row of pillars is shifted laterally. Based on the arrangement of these pillars, there will be a critical diameter (D_c) where particles smaller than D_c will move in a zigzag motion following streamlines downward the array and particles larger than D_c will bump into another streamline at each pillar, making them travel downward with an angle, Figure 5:5. DLDs have been used both for separation of RBC from WBC and for blood plasma separation.[101] The gaps in the DLD need to allow for separation of blood cells which are in the micrometre size, resulting in high flow resistance and low flow rates or modest blood cell removal. Holm and Beach *et al.* showed that by reducing depth of the array, cells can be oriented in a favourable way. Red blood cells will be sorted based on their radius (8 µm) and not the thickness (2 µm) in a shallow array, making it possible to increase blood cell removal and plasma yield.[102,103] It has also been shown that I-shaped pillars instead of circular pillars cold be used to separate RBCs from rod-shaped bacteria. I-shaped pillars made the non-spherical RBCs and bacteria rotate, giving them both an increased D_c and made it possible to increase distances between pillars and increase throughput.[104] An asymmetric DLD have been developed and it shows that both lateral and downstream gap size affects the D_c. By reducing downstream gap size, an improved removal of RBCs from diluted blood sample was shown in a device more easily fabricated than I-shaped DLDs.[105] Common for DLD based devices are that low flow rates and diluted samples often are needed to avoid clogging as the pillars are close in distance, see performance in Table 5:7.

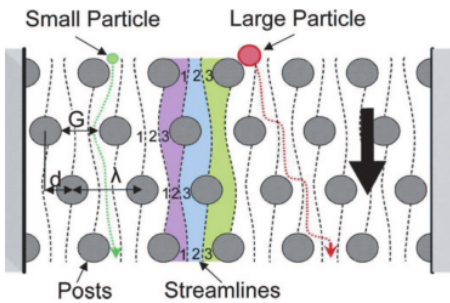


Figure 5:5: A DLD device, smaller particles moves in a zigzag motion downwards while larger particles will be bumped in into another streamline at each post. Reprinted with permission from reference [101]. Copyright (2006) National Academy of Science.

Table 5:7: Deterministic lateral displacement blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
DLD for plasma separation[101]	Cell-free	All plasma	Undiluted	0.4 µL/min	
DLD, optimised depth for RBC/parasite separation. [102,106]	97%	-	Undiluted, serum removed	0.06 µL/min	83.1% parasite recovery
DLD with I-shaped pillars[104]	100%	-	10x diluted	0.2 µL/min	
Asymmetric DLD with reduced downstream gaps[105]	>90%	-	10x diluted	0.5 µL/min	

Paper-based methods

Paper-based blood plasma separation can be regarded as a filtration method. However, plasma cannot be extracted from the paper and any the analysis has to take place on the paper. The detection method needs to be relatively simple as it should be integrated. Many times colorimetric assays are used[107-110] but also other methods like electrochemical detection have been reported[111]. To guide the blood separation, which takes place due to capillary forces, hydrophilic and hydrophobic regions are created, often through wax printing[107] or wax dipping[108,111]. By having agglutination antibodies at the separation site, blood cell removal can be improved for paper with larger pores as blood cell clusters will leak through the filter to a lesser extent.[107] Most often plasma travels horizontal through the paper but devices have been shown where paper are stacked on top of each other and plasma flows vertically through the separation paper stack to reach the detection site.[109] Paper has also been used to create flow channels in a origami way. Kar *et al.* showed an origami H-filter in paper where blood flow was aligned with a buffer flow and biomarkers were allowed to diffuse into the buffer stream.[110] Paper based blood plasma separation methods usually require only small (finger prick) volumes and the analysis function has to be simple. Since only low sample volumes are handled, detection of biomarkers present in high concentration are preferred. Example of a wax dipped paper based blood assay can be seen in Figure 5:6 and the performance of the above mentioned methods is summarised in Table 5:8.

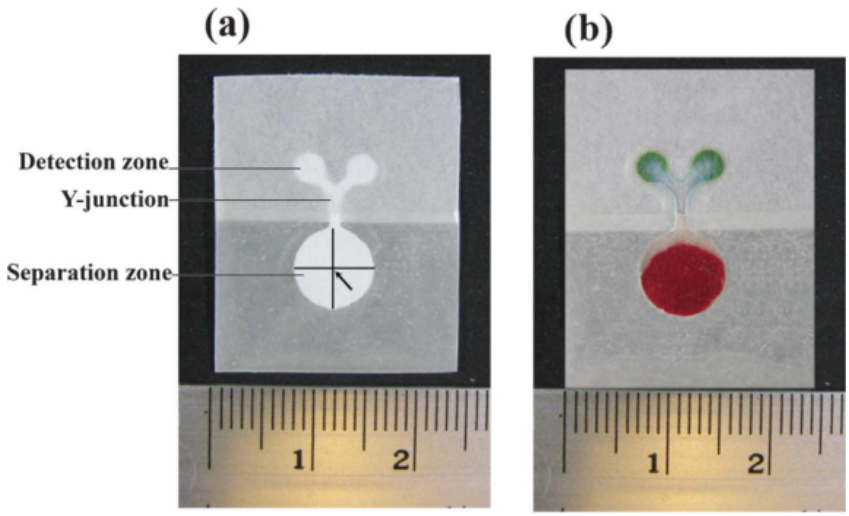


Figure 5:6: Wax dipped blood separation membrane and filter paper. When blood was added to separation site, plasma travelled by capillary forces toward detection site where colorimetric detection of total protein occurred. Reprinted with permission from reference [108]. Copyright (2012) Royal Society of Chemistry.

Table 5:8: Paper based blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Wax printed filter paper, 11 µm pores, with agglutination antibodies[107]	95%	High	Undiluted	7 µL	Integrated colorimetric detection of glucose
Wax dipped filter paper and blood separation membrane[108]	100%	-	24-55% HCT	15-22 µL	Integrated colorimetric detection of plasma proteins
Wax dipped filter paper and blood separation membrane, 2 separation sites[111]	-	-	24-60% HCT	250 µL	Integrated electrochemical detection of glucose
Blood separation filter stacked on a wax printed paper[109]	-	-	Undiluted	15 µL	Integrated colorimetric detection of liver function markers and total protein
Origami folded 2D paper device with H-filter[110]	75%	-	37% HCT	50 µL	Integrated colorimetric detection of glucose

Centrifugation

Centrifugation is the gold standard for plasma separation on the macroscale. On the microscale centrifugation can be performed in plastic a CD that can be rotated at high speed, often referred to as Lab-on-a-disc or Lab-on-a-CD (LabCD). In contrast to passive methods not requiring external actuation it is an active method requiring external rotation to generate the centrifugal force. The separation principle is the same as centrifugation at the macroscale, but using this technique there is a possibility to integrate the separation with a miniaturised assay. While the separation generally is performed the same way, the design of the disc with reaction chambers and valves to control flow of plasma varies. Already in 1992 Abaxis Inc. presented a complete POC system that within 10 minutes generated plasma, performed up to 12 different tests in addition to internal controls, calculated and communicated result.[112] Several LabCDs for blood plasma separation have been presented since then, often with focus on small volume samples.[113-115] However, Amasia *et al.* demonstrated a LabCD device which could handle a 2 mL blood sample.[116] It has been shown that bead based immunoassays can be integrated with LabCD devices as liquid and beads easily can be separated by the centrifugal forces.[114,115] Even if complete POC systems were introduced early[112], there are not many system commercially available. The reason may be expensive disposable disc and that a newly introduced platform must be more cost-effective, have higher throughput, be user-friendly, and be more accurate to replace the gold-standard.[117] Besides drawbacks as expensive disposable parts and shelf-life of the preloaded reagents onto the disc advantages are the high plasma yield generally produced in LabCDs and that plasma can be moved between different parts of the disc in a controlled way to effectively preform assays. The device performance of a few LabCDs for blood plasma separation is presented in Table 5:9.

Table 5:9: LabCD based blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Complete LabCD system[112]	-	-	Undiluted	40-100 µL	Integrated with colorimetric detection of 12 different biomarkers
LabCD for blood separation[113]	89%	40%	Undiluted	5 µL in 20 s	
LabCD with bead based ELISA[114]	-	33%	Undiluted	150 µL	Integrated bead ELISA for detection of hepatitis b
LabCD with bead based immunoassay[115]	-	-	18% HCT*	1 µL	Integrated bead immunoassay for detection of C-reactive protein and interleukin-6
LabCD for blood separation, multiplexing[116]	99.9%	37.5%	Undiluted	2 mL in 2.5 min	Porcine blood

* Calculated from whole blood = 45% HCT

Electrical separation

Dielectrophoresis (DEP) is an active separation method which relies on external electrical fields. Electrostatic forces arise because of an inhomogeneous electric field between two electrodes and this results in polarisation of the medium and particles present in the field. Depending on the relative permittivity of the particles and medium, particles will move toward regions with high electric field (positive DEP) or regions with low electrical field (negative DEP). DEP has proved useful for improving cross-flow filtration by actively unblock the branching channels and enhance the volume that could be processed.[118,119] Blood cells can also be trapped in a single channel while letting plasma continue flow forward. An electro-osmotic flow (EOF) can be generated by having a potential difference over the channel. This has been utilised to make blood flow through a main channel and in combination with a weak transverse electric field, plasma could flow through branching channels while blood cells continued in the main channel.[120] DEP and EOF have been used together with hydrodynamic separation generate a plasma zone, Figure 5:7. Blood flow through the channel was achieved by EOF and blood cells were hydrodynamically trapped in stagnant zones in dead end branches and by turning on DEP blood cells also got trapped close to the inlet. This resulted in a plasma zone located centrally in the channel where optical investigation could be done.[121] The performance characteristics for the above mentioned electrical separation methods are presented in Table 5:10.

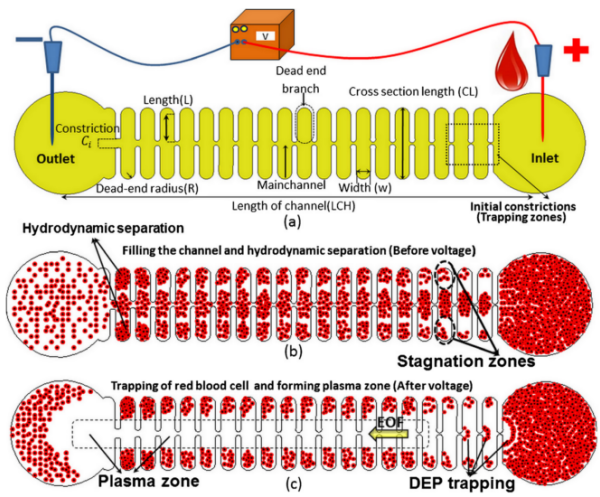


Figure 5:7: Blood moved from inlet to outlet by the means of EOF. Blood cells were hydrodynamically trapped in dead-end regions and by DEP close to inlet. Plasma region free from cells appeared along the channel. Reprinted with permission from reference [121]. Copyright (2015) Springer Nature.

Table 5:10: Electric blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Negative DEP, trapping of blood cells[118]	97%	6%	10x diluted	5 µL	
Negative DEP to actively unblock cross-flow filtration[119]	Cell free plasma*	1.1%	Undiluted	15 µL	
Positive DEP to trap blood cells	89.4%	38%**	Undiluted	150 µL	Low haemolysis
EOF, blood flow in main channel, plasma flow in branching channels due to weak transverse field[120]	Almost 100%	8-26%	16x diluted	8 µL	
EOF in main channel + trapping of cells due to hydrodynamic forces + DEP trapping of cells[121]	99%	5%	Undiluted	2 µL in 7 min	

* Data not shown

** Calculated from whole blood = 45% HCT

5.3 Acoustic blood plasma separation

Acoustic separation, or acoustophoresis, is an active microfluidic method where the acoustic radiation force is accountable for the movement of blood cells within a microfluidic channel. Acoustophoresis has been utilized for different types of blood handling by our group[51,122-133] as well as others[134-142]. This section reviews acoustic blood plasma separation methods as well as the principle behind the blood plasma separation in the papers included in this thesis.

Acoustic blood plasma separation presented by others

The fact that blood cells are affected by ultrasound was known long before the field of acoustofluidics emerged. In 1971, Dyson *et al.* showed that RBCs formed a band in the blood vessels of live chicken embryos during ultrasonication[143]. A year later, Baker showed that blood samples in a polystyrene container sediment much faster during sonication at 1 MHz[144] and enhanced sedimentation has after that been further investigated[145].

Moving over to microfluidics, Yasuda *et al.* showed already in 1997 that RBC could be focused in the centre of a quartz chamber actuated with a half wavelength standing wave.[134] Even if the separated cells were not extracted in this work, they have earlier shown a similar system where particles were extracted by putting a capillary in the chamber to withdraw the focused particles.[146] In a more appropriate setup for

separation, lipids could be separated from RBCs in a half wavelength resonance silicon/glass microfluidic chip where the separation channel had a bifurcation at the end. The RBCs had a positive contrast factor and were consequently focused to the pressure node while lipid particles had a negative contrast factor and therefore moved to the pressure antinodes along the side walls, Figure 5:8. [147] Later they also showed that RBCs, WBCs and platelets could be fractionated from a buffy coat sample in a similar design but with several outlets. Here all cells had positive contrast factor but their different sizes and acoustophysical properties resulted in different primary radiation forces acting on each cell type. By controlling the retention time in the separation channel, the cells could be separated into different bands and collected at individual outlets.[148]

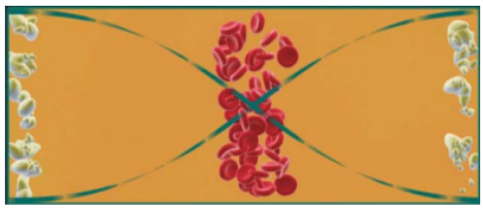


Figure 5:8: A half wavelength resonator separating blood cells with positive contrast factor from lipids with negative contrast factor. Reprinted with permission from reference [147]. Copyright (2004) Royal Society of Chemistry.

To accomplish both high throughput and high blood cell removal, an acoustofluidic blood plasma separation where four separation chips were coupled in series, each with eight parallel channels utilising half wavelength resonance was presented. Sequentially the HCT was lowered and the final plasma extracted from undiluted whole blood contained less than 1% blood cells.[149] Later, Lenshof *et al.* presented a simplified meander like separation channel still having a half wavelength standing wave which at a flow rate of 80 µL/min could generate a plasma yield of 12.5%. Blood cell removal was close to 100% and plasma was further used for off chip detection of PSA.[123] Focus of diluted blood to the middle of a half wavelength resonance has been shown in a plastic chip which is known to have moderate acoustic characteristics but is much cheaper option than silicon or glass. A blood cell removal of 98% was reported, however, driving voltages were higher than normally used for silicon and glass devices.[140]

Also surface acoustic waves (SAW) have been used for blood separation. In a SAW device interdigitated transducers transmit an acoustic wave traveling along the surface of a substrate. It has been shown that platelets could be separated from undiluted whole blood in a PDMS device. The sample was initially focused to the centre line by side

sheath flows followed by acoustic focusing of RBCs and WBCs to the channel walls while platelets (with low acoustic mobility) and plasma continued in the centre, Figure 5:9. The purity of the plasma was close to 98% with a flow rate of 0.25 µL/min.[135] In a similar setup microvesicles (MVs) were separated from concentrated RBCs (for transfusion)[136] A SAW device has also been used to isolate exosomes from blood cells, MVs and apoptotic bodies. More than 99.999% of the blood cells were removed from the exosomes when running sample at 4 µL/min.[142] In an approach for blood bacteria separation, a SAW device was used for blood plasma separation. Sample (50 times diluted at 1 µL/min) entered close to one side wall and blood cells were focused to the opposing channel side, into a sheath buffer and separated from the bacteria containing plasma which remained close to the first side wall. The produced plasma samples had a purity close to 96%.[139]

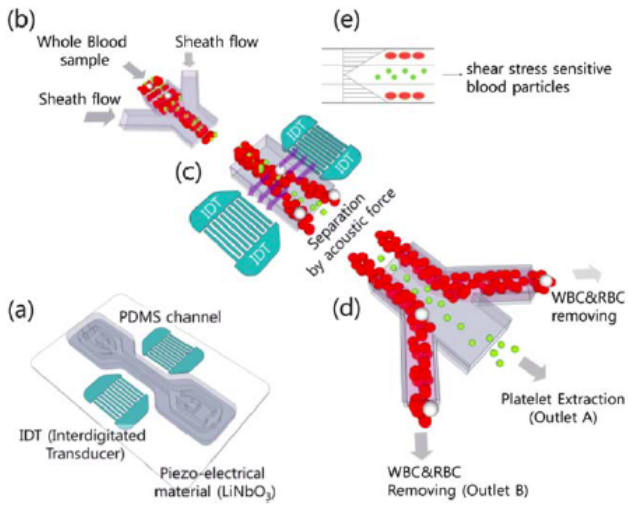


Figure 5:9: (a) A surface acoustic wave (SAW) device with interdigitated transducers to produce surface waves. (b) Blood enters the separation channel in the centre and blood cells are focused to the side walls by the acoustic force while (d) platelets and plasma can be collected from the centre. (e) Platelets, sensitive to shear stress that can cause activation, are kept from the side walls (but not the top and bottom). Reprinted with permission from reference [135]. Copyright (2011) Royal Society of Chemistry.

The approach to move blood cells over to sheath buffer has also been utilised in bulk acoustic devices. With the goal to separate bacteria from a blood sample, Ngamsom *et al.* showed a bulk acoustofluidic device with a half wavelength standing wave between side walls in a glass chip for blood plasma separation. The sample entered

on the sides (10 µL/min) while a buffer, entering in the centre, laminated the sample along the side walls. Blood cells were focused to the centre buffer while plasma and bacteria remained on the side which resulted in a blood cell removal of 99.8% but also losing most of the bacteria to the centre outlet.[138] To reach a high throughput, separation of plasma containing platelets from whole blood was performed by bulk acoustic waves in a stainless steel chamber. When introduced close to the bottom wall, RBCs and WBCs were focused toward the top wall by a quarter wavelength resonance while plasma and platelets remained close to the bottom wall. More than 80% of RBCs and WBCs were removed at a flow rate of 10 mL/min.[137]

Even if all research reviewed here do not focus on pure blood plasma separation but rather some other blood separations, their devices could probable also be used and optimised for that purpose. Further device performance can be found in Table 5:11.

Table 5:11: Acoustic blood plasma separation

Description	Blood cell removal	Plasma yield	Sample	Sample volume	Comments
Bulk acoustic silicon/glass device, four 8-channel chips coupled in serial[149]	99%	-	Undiluted	0.6 mL/min	Bovine blood
Bulk acoustic silicon/glass device with meander channel[123]	Almost 100%	12.5%	Undiluted	80 µL/min	Detection of PSA off-chip
Bulk acoustic plastic device with centre buffer flow[140]	98%	-	9x diluted	110 µL/min	34% bacteria recovery
SAW device that focuses RBCs and WBCs into side buffer flow (Figure 5:9)[135]	98%	-	Undiluted	0.25 µL/min	RBCs and WBCs separated from plasma and platelets
SAW device that focus RBCs and WBCs into side buffer flow[136]	-	-	Packed RBCs (transfusion)	-	Purification of Microvesicles from packed RBCs
Saw device, blood cells focused to side buffer[142]	99.999%	-	Undiluted	4 µL/min	Isolating exosomes from blood
SAW device that focuses blood cells to side buffer flow[139]	96%	-	50x diluted	1 µL/min	96% bacteria recovery
Bulk acoustic device in glass, blood cells focused to centre buffer[138]	99.8%	-	2x diluted	10 µL/min	10% bacteria recovery
Bulk acoustic device in stainless steel, RBCs and WBCs focused to buffer in top[137]	80%	-	Undiluted	10 mL/min	RBCs and WBCs separated from plasma and platelets

Acoustic blood plasma separation presented in paper I

The acoustic blood plasma separation channel used in the project presented in paper I has one blood inlet, four blood waste outlets and one plasma outlet, Figure 5:10. The blood sample enters through the inlet and blood cells (RBCs and WBCs) are focused by the primary radiation force towards the centre line of the channel. The three first waste outlets (A-C) are located in the centre of the channel bottom in the meander channel and focused blood cells are drawn out, from the central streamlines rich of blood cells, to sequentially lower the blood cell concentration. At a bifurcation in the end of the channel the remaining focused blood cells exit through a centre outlet (D) while plasma passes on the sides before exiting through the last outlet (E). Previous work had shown that the plasma produced by a similar separation chip could generate plasma with a cell content of $3.7x10^9$ RBCs/L and a blood cell removal close to 100% was acchived.[123] This plasma was then collected and a microarray sandwich immunoassay for detection of PSA was done to evaluate the plasma quality. In the work presented in paper I, the separation channel was modified slightly by an elongated channel (now 238 mm long instead of 224mm) and rearranged outlets to enable integration with the PSA assay manifold. Flow rates were changed and undiluted whole blood was drawn in-to the chip at a flow rate of 50 µL/min and at each waste outlet blood cells exited at 10 µL/min, resulting in a plasma production rate of 10 µL/min. Syringe pumps pulled fluid through all the waste outlets and from the PSA assay manifold, connected to the plasma outlet, while the blood sample input was drawn from an open tube. Because of optimisation of flow rates, this device had a volumetric plasma yield of 20% compared with 12.5% in the previous design, but the produced plasma contained slightly more RBCs, now $4.2x10^9$ RBCs/L. This cell number is still below $6x10^9$ RBCs/L which is recommended for clean plasma by Council of Europe[150]. The WBCs have shown to have basically the same acoustic mobility as RBCs and are believed to be focused to the same extent as the RBCs[133]. However, the WBC content in the plasma was not investigated. Blood cell removal efficiency was not calculated as cell concentration in the input blood sample was not measured, but since a blood sample typically has $5x10^{12}$ RBCs/L the blood cell removal was likely around 99.9%.

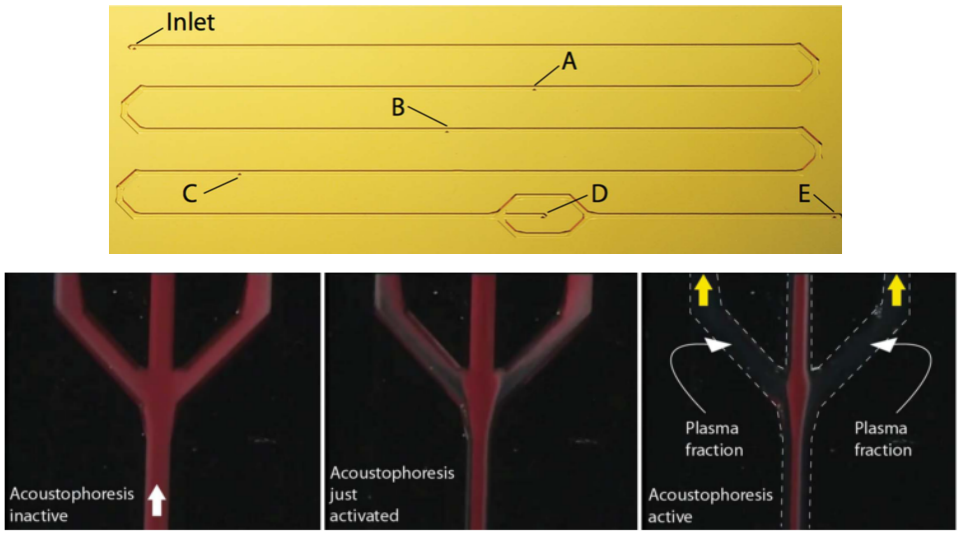


Figure 5:10: Top: A meander channel etched in silicon with one inlet, four blood cell waste outlets (A-D) and one plasma outlet (E). Bottom: Images of the bifurcation in the end of the channel where remaining blood cells are focused to the centre and plasma continues on the sides. Images from left with no ultrasound, ultrasound just started and ultrasound is active and all remaining blood cells are focused in the central outlet. Reprinted with permission from reference [125], (paper I). Copyright (2013) Royal Society of Chemistry.

The integration with a PSA assay presented in paper I

The major step in this project was that the plasma separation was integrated with the PSA assay to demonstrate a possible route towards a POC system. This PSA assay was previously presented in a manual bench top configuration[123,151] and was in the project presented in paper I optimised to a more automated and integrated flow through approach. The capturing antibody was spotted on porous silicon to form an array of 600 spots. After initial wash and blocking of the surface, the porous silicon chip was placed in a milled plastic manifold that was connected to the plasma outlet of the plasma separation chip, Figure 5:11. By having a flow of plasma over the porous silicon chip instead of just a drop incubated on top as in previous manual assay, the binding time could be reduced to 15 minutes instead of 60 minutes due to the continuous convective transport of new analyte and likewise by washing the array under constant buffer flow the washing time could be reduced from 15 to 5 minutes. The last step before detection, binding of detection antibodies, was done manually off-chip. The reason for not integrating this last step was the higher amount of antibodies that would have been needed for flow incubation of the PSA microarray. This partly integrated system could detect PSA within the clinical relevant range (1.7-100 ng/mL).

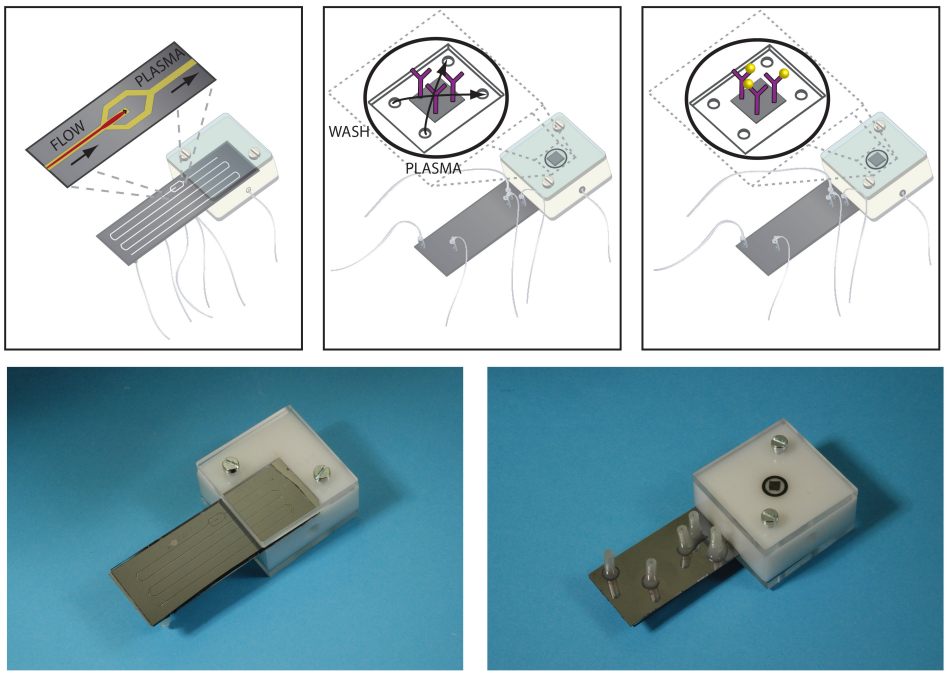


Figure 5:11: Top left: blood cells are removed in the plasmapheresis chip which is mounted in a plastic holder so that the plasma can continue to the assay chamber. Top middle and right: Plasma is perfused over the antibody arrayed porous silicon chip with and PSA is captured by the antibodies. Bottom: Photograph of the integrated device. Reprinted with permission from reference [125], (paper I). Copyright (2013) Royal Society of Chemistry.

Acoustic blood plasma separation presented in paper II

Even though the separation chip in paper II was intended for blood cell and bacteria separation, it actually also performed a blood plasma separation. The separation chip was a meander channel with one inlet, four waste blood cell outlets and one plasma outlet, similar to the chip in paper I. At a sample flow rate of 80 µL/min a syringe perfused blood through the chip and blood cell waste was drawn out by syringe pumps (at 20, 20, 15 and 15 µL/min) while the plasma outlet (10 µL/min) was open ended, to enable integration with an acoustic trap for bacteria concentration after which the enriched bacteria were released into a PCR chip for identification of the bacteria species. The integrated system will be explained further in section 6.3. Briefly, the volumetric plasma yield produced with these settings was 12.5% and the blood cell concentration in the plasma sample was 0.9±0.6% compared to the sample in, corresponding to a blood cell removal of 99%. The blood cell removal presented here may be difficult to compare with the blood cell removal presented in paper I as different cell counting

methods have been used (Coulter counter vs. flow cytometry) and as only the cell content in plasma was measured in the work described in paper I. The design in the project presented in paper I could not be used as we had to make place for a Peltier element underneath the chip in addition to the piezoelectric transducer. The Peltier element made it possible to control the temperature, resulting in more stable separation as temperature changes means changes in speed of sound of the sample and thereby in resonance frequency. The capacity of the acoustic trap to concentrate bacteria was highly dependent on the RBC content in produced plasma. If RBCs were not efficiently separated they would easily fill up the trap and limit the time we could run the system. Because of this and the increased variations in HCT when working with clinical samples, we had to dilute the blood samples to 70% blood to ensure sufficient RBC removal independent of the blood donor.

Acoustic blood plasma separation presented in paper III

The goal in the project presented in paper III was to focus blood cells to the centre of the channel and by image analysis determine the area that the packed blood cells cover compared to the area covered by plasma, and correlate this to the actual haematocrit. This can be regarded as a type of blood plasma separation, even if the chip only consists of a straight channel without the possibility to extract the plasma separately. Because of this, the purity of the plasma was not further investigated. It should be noted that this configuration gave visual access to the clarified plasma fraction in the chip, e.g. for optical assays. A detailed description of the presented haematocrit measurement method can be found in section 7.3.

Acoustic blood plasma separation presented in paper IV

In the project presented in paper IV bacteria were separated from blood cells, but in an optimised setup to increase bacteria recovery as compared to the separation chip presented in paper II. As in paper II, this is essentially also a blood plasma separation, although the separated plasma is diluted. The separation chip comprised just one straight channel with bifurcation at the inlet and outlet compared to the meander like channel with several outlets used in the work presented in paper II for blood bacteria separation. Diluted blood (1-20%, vol./vol.) enters through the side inlets and is laminated by a buffer solution via the centre inlet, Figure 5:12. More details about the importance of the buffer composition to optimise bacteria recovery can be found in section 6.3. By actuating a half wavelength standing wave between the side walls, blood cells were focused to the centre of the channel and the centre buffer, while plasma and the bacteria, not sufficiently large to be affected by the primary radiation force, remained on the sides. This way of separating plasma from blood cells minimises the loss of plasma and biomarkers. An input blood sample flow rate of 200 µL/min, a buffer flow of 300 µL/min, centre flow out of 200 µL/min and plasma flow out of 300 µL/min theoretically results in all plasma exiting through the plasma side outlet. However, diluted samples are required to maximise plasma recovery as higher blood cell

concentration will lead to more hydrodynamic interaction, meaning that the blood cells moving to the centre drag along a layer of plasma around them into the centre. Performance for the blood plasma separation described in paper I-IV are summarised in Table 5:12.

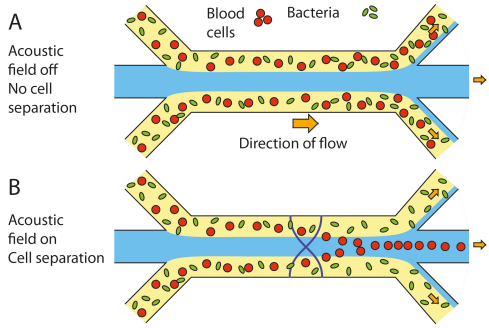


Figure 5:12: A: A diluted blood sample containing bacteria is laminated to the sides by a centre buffer. B: Blood cells are focused to the centre by the acoustic radiation force. Smaller bacteria, not as affected by the radiation force, continue together with the plasma along the sides. Bacteria and plasma are slightly diluted by the centre buffer in the end as the split ratio is set to have a small amount of buffer going out on the sides. This is done to maximise bacteria recovery and not allow any plasma with bacteria to be lost through the centre outlet.

Table 5:12: Acoustic blood plasma separation from work presented in paper I-IV

Description	Blood cell removal	Plasma yield	Sample	Sample throughput	Comments
Paper I: Bulk acoustic silicon/glass device with meander channel	99.9%*	20%	Undiluted	50 µL/min	Integrated PSA detection
Paper II: Bulk acoustic silicon/glass device with meander channel	99.1%	12.5%	Diluted to 70%	80 µL/min	Bacteria detected from spiked and clinical samples
Paper III: Bulk acoustic glass device	-	51% for 45%HCT**	Undiluted	2.7 µL***	Used for HCT measurements
Paper IV: Bulk acoustic glass/silicon device	99-99.9%	Likely close to 100% but diluted 1-20%	5-100x diluted	100-400 µL/min	Bacteria recovery 75-99.7%

* calculated from whole blood = 5*1012 RBCs/L
** For 45% HCT is 49% of the area covered with blood cells
*** Volume of channel.

5.4 Summary and discussion

Miniaturised detection assays have the advantage of only utilising small sample volumes and reagents as well as being faster than conventional assays because of the short diffusion distances. However, without integrated sample preparation and stored reagents they will not be convenient for the operator. If small volumes need to be pipetted, the hands-on time may be long even if the assay itself can be cheap and fast. Operator dependent errors and variations may also arise form pipetting differences, time from drawing the sample and the way the blood sample is stored prior to use. Because of this, an integrated sample preparation is preferable so that the assay can be performed in a repeatable manner and immediately after sampling.

Generating clinical plasma

Because of the small channel dimensions, many microfluidic blood plasma separation methods consume small sample volumes and are most appropriate for finger prick volume blood samples. This includes for example filtering methods, paper-based methods, deterministic lateral displacement and electrical methods. Centrifugation based microfluidic methods often handle small volumes as well, but have the possibility to process larger samples. This requires that the biomarker of interest exists in a sufficiently high concentration to generate a statistically accurate result. If collecting the blood through a finger prick one must confirm that the levels of the biomarker are comparable to the levels in venous blood if that is the standard, which is not always the case.[152]

Hydrodynamic, inertial and acoustic methods can all work in a continuous manner with reasonable high flow rates so that larger volumes can be processed. Many of the reported hydrodynamic methods for blood plasma separation work with highly diluted blood samples which decreases the sensitivity of the following biomarker detection as also the marker is diluted. A few hydrodynamic methods have however been reported to work on undiluted[88,94] or close to undiluted samples[89]. Biomarkers as cell-free DNA, glucose, hCG (human chorionic gonadotropin, a pregnancy indicator) and total protein have been measured from these high concentrated blood samples.[88,94] However, validation of the highly diluted samples has also been done with measurement of total protein, protein profile study and protein cancer markers.[83,85,86] Hydrodynamic methods working on small undiluted samples, integrated with HCT measurement and ELISA for cardiovascular disease markers, have also been presented.[80]

Inertial methods often work at very high flow rates to achieve the inertial effect in microfluidic channels. But they often also require highly diluted samples. The high flow rates can compensate for the dilution but subsequent concentration step of the biomarker may be required to avoid decrease of sensitivity of the detection.

Acoustics blood separation methods have been demonstrated both on diluted samples[138-140,153] (paper II and IV) and undiluted samples[123,125,135-137,142,149] (paper I and

III). Utilising surface acoustic waves (SAW) has the advantage that simple PDMS structures can be used, compared to the more expensive silicon or glass devices that are preferred when working with bulk acoustic waves. However, they often need very low flow rates and sometimes also diluted samples[135,139,142]. High throughput bulk acoustic devices have been presented but the extent of blood cell removal was modest.[137] Bulk acoustic devices with relatively high throughput on undiluted whole blood and high blood cell removal have previously been described[123] and shown in paper I. The plasma yield in paper I corresponds to almost half of the plasma in the initial blood sample and it may be possible to increase the plasma yield a bit more, but some of the plasma need to go with the blood cells to avoid flow disturbance by a highly viscous RBC fraction. To ensure performance over time, temperature control was added to the acoustic blood plasma separation in the sepsis project described in paper II. However, slightly diluted blood samples were used to ensure high blood cell removal regardless of variations in blood donor HCT.

In several of the reported acoustic plasmapheresis systems blood cells are moved into a buffer stream and theoretically all plasma left through the plasma outlet, sometimes diluted if some of the central buffer flow was set to leave through the plasma outlet. Increasing plasma yield is of great interest when the biomarker of interest is present at low concentrations, like for example bacteria in sepsis blood samples where there can be as few as 1-100 bacteria/mL of blood. Moving blood cells over to a buffer flow was demonstrated in paper IV to increase bacteria recovery as compared to paper II. The biomarker recovery may vary when this approach is used depending on cell concentration, since blood cells will hydrodynamically draw surrounding plasma with them into the central buffer flow. Theoretical estimates indicate that the cell concentration starts to have an impact when particle volume fractions are above 0.001 and when they exceed 0.01 hydrodynamic interactions dominates.[154] This means that if aiming at high biomarker recovery with this kind of setup, blood should be diluted to at least 1% HCT (~45x dilution).This problems is clearly seen in paper IV where bacteria recovery drops for blood diluted five times (~9% HCT) compared to 100 times diluted blood (~0.45% HCT). The analyte may also diffuse between parallel flows, making the relation between the analyte diffusion constant and flow rate important.

Depending on detection method, the blood cell removal is important. A blood sample with only 1% of the blood cells remaining still contains around 50 million blood cells/mL. This may be sufficient for some detection methods but not for all. When integrating plasma separation with detection methods the cell contamination level needs to be considered. Another thing to consider when working with microfluidics is the high surface to volume ratio which increases the risk of considerable unspecific adsorption of biomarker to surfaces. The effect would be more crucial when a smaller sample is run compared to a bigger. Surface treatments can be used to minimise this effect. When running larger undiluted blood samples through an acoustic device, surfaces are probably blocked early on by the huge amount of plasma proteins. However, the biomarker recovery needs to be tested in every single case.

5.5 Integration towards point-of-care systems

Paper based blood plasma separation is only convenient when integrated with detection methods and many integrated systems have been reported. They are often combined with simple colorimetric assays and the biomarkers detected are often present in relatively large concentrations. Colorimetric detection of glucose[107,110], total protein[108,109] and liver function markers[109] integrated with paper-based blood plasma separation have been reported. Also electrochemical detection of glucose has been shown integrated to a paper-based device[111]. Paper based methods utilise small finger prick volumes of blood samples and they are often simple in handling, making them promising for POC systems. They may also be useful in low resource areas and for use on the field when not having a highly technical medical laboratory close by.

Microfiltration and sedimentation methods for blood plasma separation also utilises small sample volumes and have been demonstrated integrated with immunoassay for IgG[155] and biotin[73] detection as well as integrated with commercial glucose stripe for glucose[71] detection. Based on hydrodynamic forces, blood plasma separation has been integrated with multiplexed immunoassay for detection of cancer markers[83] as well as HCT measurements and ELISA for cardiac troponin T. detection[80]. All these methods use small amounts of blood for different types of biomarker detections within the same device. The amount of plasma produced just covers the assay region and flow incubation, as presented in paper I, is not utilised. This type of device can be used as disposable parts in combination with a bench-top or handhold unit for the detection and analysis of the result. It is important that the disposable parts have an extended storage life time, preferably with dried reagents. This is not often reported, but a bead filter with dried antibodies was shown to keep 81% of the antibody activity during 60 days of storage.[155]

LabCD devices for blood plasma separation have also been demonstrated with integrated detection functionality. Both bead-based immunoassays[114,115] and multiplexed colorimetric assays[112] have been integrated to LabCD devices. Similar to the above, a POC system based on centrifugal forces can be performed in disposable parts in combination with a driving and detection unit. Preferably, the LabCD should be able to handle storage time with dried reagent.

The blood plasma separation devices presented in paper I-IV are all in glass/silicon or just glass. This is because of the good acoustic properties in these materials, which give sufficiently high acoustic energies to make them useful for diluted as well as undiluted blood samples. For an industrial point of view this might make them too expensive to be provided as a disposable component and therefore more appropriate for multi-use applications. In paper II is it shown that it is possible to sterilise the separation chip in-between runs and avoid DNA cross-over from different samples. Bulk acoustic device performing blood plasma separation in plastic channels has been reported[140] and it may be possible to make a disposable device. However, the performance is not yet

comparable to bulk acoustic device in silicon or glass. Acoustic blood plasma separation could preferably be used when larger volumes, in the mL range, are needed. As shown in paper I, the time for an immunoassay can be shortened by flowing plasma over the capturing site. Possibilities of leading plasma over multiple detection sites also appear with larger volume plasma produced. For the case with bacteria detection in sepsis blood samples, a larger volume has to be processed to ensure isolation of enough bacteria for detection as presented in paper II and IV. In conventional blood culture a minimal volume of 5 mL blood is cultured to ensure the required limit of detection of 1 CFU/mL.

If not aiming for disposable acoustic separation chips these can still be integrated by interfacing to detection units, similar to paper I. Acoustic plasma separation chip can also be interfaced with multiple units like a concentration unit followed by a detection unit, as in paper II.

When detecting biomarkers present in high concentration in the blood sample, paper-based or microfiltration methods can preferably be used because of their simplicity and use of small finger prick volumes of blood samples. For biomarkers present in low concentration, a continuous blood plasma separation method based on hydrodynamic, inertial or acoustic forces could be used. In this way a larger volume can be processed to ensure a large enough sample to get a statistically certain result. Hydrodynamic, and inertial methods are passive methods which can be used in a more simple setup, but they often require diluted samples. Electric methods are often restricted to small volumes and slow flow rates even if continuous, making them more suitable for detection of high concentration biomarkers. Acoustic methods, which can run continuously on both diluted and undiluted samples, would be useful for detection of rare biomarkers as well as for integration to flow through assay. Because of the material requirements, they would not be suitable as disposable parts, but rather as a bench top device which is reused.

6. Blood bacteria separation

When bacteraemia results in the severe inflammatory response, called sepsis, immediate antibiotic treatment is crucial for the patient. In its most severe case, a patient can get into septic shock which leads to dangerous low blood pressure and organ failure. In these cases, every hour of delayed antibiotic treatment is associated with 8% less chance for survival and fast as well as correct antibiotic treatment is important.[156] In case of sepsis, bacteria can often be found in the blood and first when they are detected and identified, correct antibiotic treatment can start. Before that, broad-spectrum antibiotics is used for treatment if sepsis is suspected.

6.1 Why separation of bacteria from blood?

The amount of bacteria in a blood sample is low, as few as 1-100 colony forming units (cfu) of bacteria can be found in one mL of blood which contains billions of blood cells.[157-159] Normally blood is cultured (5-130h) to bring the concentration up before identification by gram staining, sub culturing, PCR or MS. As mentioned in chapter 2, there are ways of identifying bacteria directly without culturing. One example is SeptiFast (Roche Diagnostics, GmbH, Mannheim, Germany), a commercially available PCR method where bacterial nucleic acids is extracted direct from a patient blood sample. Blood cells are lysed prior to DNA extraction to reduce the complexity

of the sample. However, this direct PCR system has around 100 manual handling steps and the risk for contamination or human error is big. This has so far prevented it from replacing blood culture. Another approach for bacterial identification could be separation of bacteria from the blood cells to reduce the complexity and background, followed by PCR detection. If a separation method with high recovery could be integrated with detection and automated, a faster, more stable and trustable system for bacteria identification could be developed.

6.2 Microfluidic blood bacteria separation

Microfluidic ways of separating blood cells and bacteria could be promising for a new bacteria identification system. This method should be able to handle large enough blood volume to ensure bacteria identification even in the cases with very low bacteria concentration. For the same reason, bacteria recovery needs to be high. This opens up for the use of continuous microfluidic devices to reach the good separation performance as well as the possibility to process larger blood volumes. Several continuous microfluidic methods for separation of bacteria from blood have been reported, including dielectrophoresis[160-163], inertial lift forces[96,164-166], Fåhraeus effect[167,168] and acoustic methods[138-140,153](paper IV). Dielectrophoresis can generate both high bacteria recovery and blood cells removal but suffers from the need of highly diluted blood samples and low flow rates.[160,161] As consequence, processing 1 mL undiluted blood sample would that take from 28 up to several hundreds of days, which is not a reasonable time. Inertial separation of bacteria from blood can give blood cell removal just over 90%, which still leaves much blood cells in the bacteria fraction.[96,166] To process 1 mL undiluted blood would take from 17 h up days, which still is too long. A system utilising margination for blood bacteria separation with the goal to remove bacteria and inflammatory components (WBCs and platelets) from blood as a sepsis treatment instead of bacteria identification has been suggested.[167] The Fåhraeus effect only moves deformable RBCs while the more rigid WBCs and platelets are unaffected, Figure 6:1. This device could process 1 mL undiluted whole blood in the more reasonable time of 1 h but with a bacteria recovery of 80% and blood cell removal of 83.2% which not may be sufficient for bacteria identification.

Later a faster system with higher RBC removal but less bacteria recovery was presented which could process 1 mL undiluted whole blood in 33 min.[168] This system was also parallelised to a 16 channel system where bacteria recovery dropped even more. A 32-channel device was also demonstrated to run on undiluted whole blood making it possible to process 1 mL in 12.8 minutes, but no separation data was shown. As the Fåhraeus effect does not affect the more rigid WBC, they would still be present in the produced plasma which could interfere with detection. Performance of different microfluidic devices for separation of bacteria from blood is presented in Table 6:1.

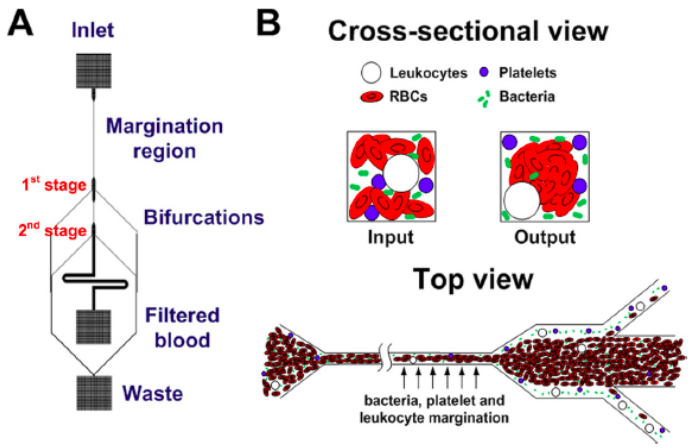


Figure 6:1: A: Microfluidic chip for removal of bacteria, WBCs and platelets from blood as a sepsis treatment. B: Deformable RBCs migrated to the centre while the more rigid WBCs, platelets and bacteria were unaffected. Top view of the separation where the RBC free layer was extended by constriction followed by expansion. Reprinted with permission from reference [167].

Table 6:1: Microfluidic separation of bacteria from blood

Description	Blood cell removal	Bacteria recovery	Sample	Sample volume	Time to run 1 mL undiluted blood
Dielectrophoresis[160]	98%	80%	1000x diluted	1 µL/min	694 days
Dielectrophoresis[161]	100%	87.2%	20x diluted	0.5 µL/min	28 days
Dielectrophoresis[162]	-	30%	2x diluted	35 µL/min	2.4 days
Dielectrophoresis[163]	-	97%	20x diluted	10-20 µL/h	83 days
Inertial[164]	-	-	22x diluted	18 µL/min	20 h
Inertial[96]	90%	>80%	200x diluted	200 µL/min	17 h
Inertial[165]	-	75%	4x diluted	-	-
Inertial[166]	92% (71%)	76% (73%)	Undiluted	0.5 µL/min (1 µL/min)	1.4 days (17 h)
Fåhraeus effect[167]	83.2%	80%	Undiluted	16.7 µL/min	1 h
Fåhraeus effect (16 channel; 32 channel)[168]	90 % (-, -)	70% (50-60%, -)	Undiluted	30 µL/min (-, 78 µL/min)	33 min (-, 12.8 min)

Affinity-based extraction of bacteria from blood samples has also been performed on the microscale. Antibodies[169,170] as well as bacteria binding proteins[171] and synthetic ligands[172] have been used to acquire bacteria affinity on beads. Simple microfluidic structures can be used to move bacteria over from blood to a buffer with the help of bacteria binding to magnetic beads.[169] This has been used to develop blood cleansing device for sepsis therapy[171,172], which possibly also could be used with the aim to recover the bacteria for identification. These beads were modified to bind a broad range of bacteria and had high throughput as the goal was to process the whole blood volume. To reach high bacteria recovery several passes through the system was necessary. Magnetic beads with capturing ability have been used in miniaturised fluidised beds where magnetic forces and drag forces were balanced to keep the bed fluidised while perfused with sample.[170] By studying the growth of the bed when bacteria were captured and cultivated, a low limit of detection as well as simple detection methods was realised. Yet only tested on bacteria detection from food samples, it can possibly be a favourable method for blood samples because of the continuous flow approach combined with enrichment by cultivation. Affinity-based methods generally have lower capturing efficiency for complex samples, like whole blood, than buffer samples and may need dilution or removal of blood cells prior to binding to reach high bacteria recovery for bacteria identification. Bacteria and beads need to come in close contact with each other to ensure capturing. To process large volumes, huge amount of beads must be added and when sorted out, only a few of them will bind a bacterium.[173]

6.3 Acoustic blood bacteria separation

Acoustic blood bacteria separation presented by others

Both SAW[139,174] devices and bulk acoustic devices[138,140] have been reported for blood bacteria separation. SAW has proven to give high bacteria recovery but highly diluted blood samples in combination with low flow rates makes it unsuitable for running larger volumes as it would take over a month to run 1 mL undiluted blood.[139] A bulk acoustic device, with similar approach as in paper IV, has shown good blood cell removal and throughput but very low bacteria recovery, Figure 6:2.[138] Bulk acoustic device utilising plastic channels has also been reported with relatively high bacteria recovery and throughput but also with low bacteria recovery.[140] Performance of acoustic devices for blood bacteria separation is presented in Table 6:2.

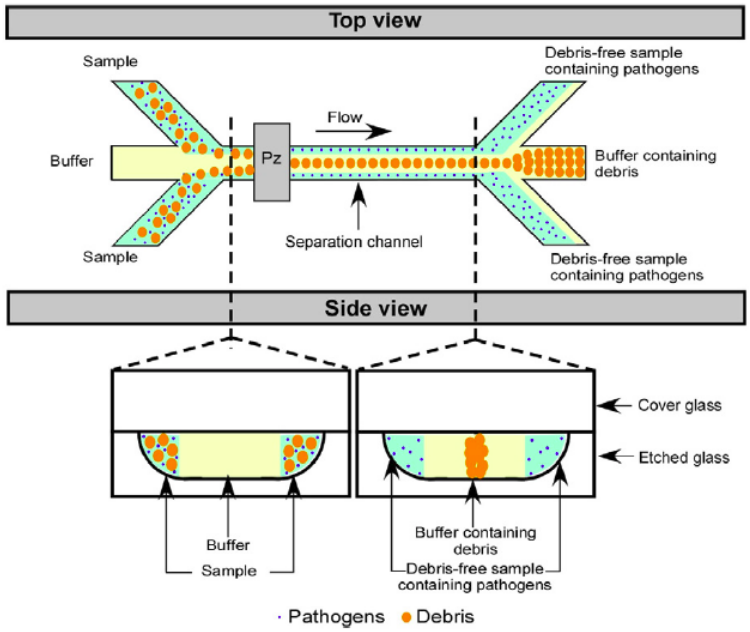


Figure 6:2: Top view: Blood sample containing bacteria was laminated along the sides by a centre buffer. Blood cell, which are bigger than the bacteria, were focused by the primary radiation force towards the centre as a result of the half wave standing wave over the width. Plasma containing bacteria could be isolated on the sides. Side view: cross-section of the channel without and with ultrasound actuated. Reprinted with permission from reference [138]. Copyright (2016) Elsevier B.V

Table 6:2: Acoustic blood bacteria separation

Description	Blood cell removal	Bacteria recovery	Sample	Sample volume	Time to run 1 mL undiluted blood
SAW device[174]	95.65%	-	WBCs and bacteria	-	-
SAW device[139]	96%	96%	50x diluted	1 µL/min	35 days
Bulk acoustic glass device[138]	99.8%	10%	2.2x diluted	10 µL/min	4h
Bulk acoustic plastic device[140]	98%	34%	9x diluted	110 µL/min	1.4 h

Acoustic blood bacteria separation presented in paper II

A meander channel was used for the separation of blood and bacteria in this project. Photograph of the separation chip can be seen in Figure 6:3 (A) together with the acoustic trap for enrichment and PCR-chip. The integrated system is described further in section 6.4. Blood entered the separation chip and blood cells were focused to the centre. Focused blood cells were removed through outlets located in the centre of the bottom wall and the HCT was sequentially decreased. The third blood cell outlet was located in a bifurcation region where remaining blood cells left through the centre waste outlet while plasma continued on the sides, Figure 6.3 (B). Blood was slightly diluted (to 70% blood) and was run at 80 µL/min, meaning that it took 14 min to process 1 mL undiluted blood. However, the bacteria recovery was low, only 11 %. This recovery is reasonable if bacteria were uniformly distributed in the sample and only 12.5% of the flow left through the plasma outlet. Still, this design managed to separate out bacteria from clinical samples. These bacteria were then concentrated and detected in an integrated system, see section 6.4.

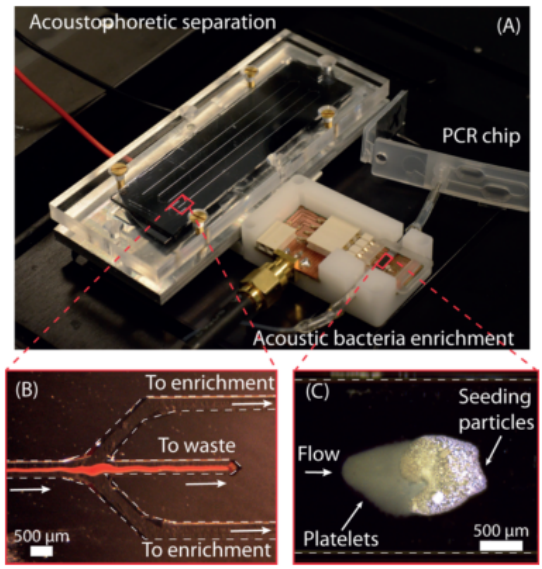


Figure 6:3: (A): Blood diluted to 70% entered the temperature controlled meander separation channel at 80 µL/min. Centrally focused blood cells were removed through three centred bottom outlets (at 20, 20 and 15 µL/min). (B): In a bifurcation at the end, the last focused blood cells were removed (at 15 µL/min) while plasma containing bacteria left the separation chip at a flow of 10 µL/min. (C): Produced plasma continued into the acoustic trap where bacteria were enriched with the seeding particles (and platelets). Reprinted with permission from reference [125], (paper I). Copyright (2013) Royal Society of Chemistry.

Acoustic blood bacteria separation presented in paper IV

In an attempt to increase the bacteria recovery compared to the recovery described in paper II a new fluidic setup was tested, Figure 6:4 A-B. The blood sample was laminated along the sides by a centre buffer in a channel with half wavelength resonance over the width of the channel. By moving the blood cells over to the pressure node in the buffer a higher plasma yield as well as bacteria recovery were reached. This, however, required diluted blood samples to minimize the hydrodynamic interaction where blood cells drag surrounding liquid with them into the centre, Figure 6:4 D. When diluting blood 100 times 99.7% of the bacteria were recovered but when diluting the blood sample 5 times only 90% of the bacteria were recovered. In an attempt to maximise the blood cell removal, blood was diluted 5 times and the flowrate lowered from pervious 400 µL/min to 100 µL/min. This resulted in a blood cell removal of 99.99% but bacteria recovery dropped to 75%. Bacteria were affected by the acoustic force in a greater extent when the flow rate was lowered, meaning that some of them may enter the central buffer resulting in a recovery drop. Depending on the settings, it would take 12.5 to 4 h to process 1 mL undiluted blood, which would be a reasonable time for a separation step in a diagnostic setup. Essential for this system to work was the acoustic impedance of the centre buffer. A relocation of fluid would occur if the acoustic impedance of the blood is higher than the acoustic impedance of the buffer, Figure 6:4 C.[175,176] The result would be that the acoustic radiation force works on the entire blood sample which is focused to the centre resulting in a very good blood cell removal but no bacteria recovery. To increase the acoustic impedance of the centre buffer, 30% density media (Histopaque 1077, Sigma-Aldrich) was added to reach the acoustic impedance of the highest blood concertation used.

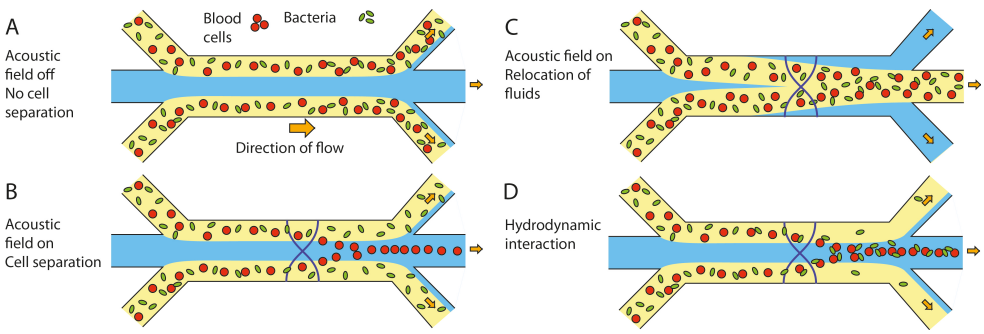


Figure 6:4: A: Blood laminated along the side by a centre buffer, no ultrasound on. B: Blood cells was focused to the centre when centre buffer was impedance matched. C: Without impedance matching of centre buffer, entire blood sample was focused to the centre. D: Plasma containing bacteria was dragged along with the blood cells towards centre when having high blood concentrations.

Table 6:3: Acoustic blood bacteria separation presented in paper II and IV

Description	Blood cell removal	Bacteria recovery	Sample	Sample volume	Time to run 1 mL undiluted blood
Paper II	99.9%*	11%	1.11x diluted	80 µL/min	14 min
Paper IV	99.9%	99.7%	100x diluted	400 µL/min	4 h
Paper IV	99.99%	75%	5x diluted	100 µL/min	50 min
Paper IV	99%	90%	5x diluted	400 µL/min	12.5 min

*Calculated from average cell concentration

6.4 Towards sepsis diagnostics

A promising blood bacteria separation on its own is not enough to reach the goal of a sensitive, fast and automatic system for sepsis diagnostics. As many microfluidic methods need diluted samples to achieve high bacteria recovery, large volumes need to be processed. To adapt the output of the blood bacteria separation to a miniaturised detection system, concentration of the bacteria as well as volume reduction may be necessary. A few attempts have been made to integrate microfluidic blood bacteria separation with detection in the development of a tool for pathogen identification in blood samples. Capturing of bacteria on surface modified pillars[177] and separation through dielectrophoresis[178] have both been integrated with PCR detection but have not been tested on clinical samples.

Integration with enrichment and detection in paper II

The integrated system presented in paper II was tested both on spiked samples and on clinical samples. Blood samples of 1 mL were diluted to 1.43 mL before processed in the acoustic separation where blood cells were removed while plasma containing bacteria continued to the acoustic trap. The acoustic trap, a glass capillary with a small transducer underneath to create a local acoustic field, was preloaded with 12 µm seeding particles to enable capture the smaller bacteria. After perfusion of the whole sample through the separation chip and acoustic trap, the trapped cluster was washed with buffer to remove plasma which interfered with the PCR detection, Figure 6:5. Trapped bacteria were then released into the PCR chip connected to the acoustic trap and the PCR chip was placed in the PCR machine for detection. One sample could be processed in less than 2 hours from sampling to identification.

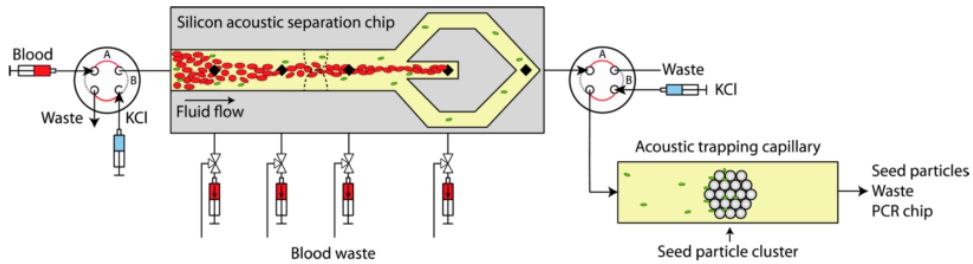


Figure 6:5: The blood sample entered the separation chip through a valve and the focused blood cells were removed while bacteria suspended in plasma continued through a second valve and into the acoustic trap. The acoustic trap was preloaded with seeding particles and bacteria were trapped in-between the seeding particles over the small transducer located underneath the glass capillary. Wash buffer was used to remove plasma from the acoustic trap before the trapped bacteria were released into the PCR chip for detection. Reprinted with permission from reference [153], (paper II).

Four different PCR chips were used for detection of four different bacteria: *Pseudomonas* spp., *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. The sensitivity of the integrated system was tested with blood samples from healthy donors spiked with *Pseudomonas putida* and detected by *Pseudomonas* spp. chip. The sensitivity of the integrated system was around 1000 bacteria/mL which is reasonable as around 11% of bacteria are recovered in the separation step, 95% recovered in the trapping step and that the limit of detection for the PCR chip itself was around 100 *P. putida*/mL. The sensitivity of the integrated system also depended on the different PCR chips used, where *S. aureus*, *S. pneumoniae* and *E. coli* had a limit of detection of 3, 2 and 400 cfu/chip.

During the run of clinical samples, each sample was tested for *E. coli*, *S. aureus* and *S. pneumonia*. Out of 57 processed samples only 7 were positive in blood culture, 4 for *E. coli* and 3 for *S. aureus*. The integrated system barely detected two of the four *E. coli* samples and these where the samples with shortest blood culturing time, which may indicate that they had a higher bacteria concentration from start. None of the *S. aureus* which were positive in blood culture were detected in the integrated system, which may be due to low number of bacteria or properties affecting the separation. Such properties could for example be clustering of bacteria so that they appear to be bigger particles and are sorted away in the separation step.

Outlook

The development of a microfluidic automated system for bacteria identification from blood samples would speed up the time from sampling to treatment with targeted antibiotics. Fast identification can be crucial for the patient as initial treatment with broad-spectrum antibiotics is not working in 20-34% of the cases.[179,180] The extensive use of broad-spectrum antibiotics also leads to increased antibiotic resistance, which is a big threat for patients with infectious diseases. For many of the microfluidic methods utilised for blood bacteria separation the time consuming culturing step will still be needed because of their low recovery and throughput.

The limited clinical study in paper II indicates that this integrated system could detect the clinical samples which had higher bacteria concentration but not yet the ones with very few bacteria. The vast majority of the bacteria is probably lost in the separation step which only has 11% bacteria recovery. By integrating the optimised blood bacteria separation, with bacteria recovery of 99.7%, the integrated system probably would get lower limit of detection. However, the improved bacteria separation described in paper IV means that larger volumes need to be processed because of the dilution. An improvement of the sensitivity of the PCR itself would also increase the overall sensitivity. A device for bacteria identification to be used in clinics should use a multiplex PCR to be able to detect a broad range of pathogens at once.

7. Haematocrit measurement

Haematocrit (HCT) is routinely measured to investigate a patient's blood status. It is defined as the volume percent of the blood that is red blood cells (RBCs) and reveal dehydration or blood loss due to internal bleeding as well as conditions such as anaemia or polycythaemia. It is today mainly performed by automated cell counters in centralised lab facilities but can also be performed by centrifugation methods such as the microhaematocrit, which was described in section 2.3.

7.1 Microfluidic haematocrit measurement

The benefit of using microfluidics for HCT measurements may not be to present a new way of measuring it that can replace centrifugation or automatic cell counters, but rather the possibility to integrate the method with other diagnostic microfluidic tests. Various microfluidic methods for HCT measurements have been presented, including an optical readout from a LabCD device that within a few minutes can give a HCT value from minimal amounts of blood.[181,182] Another LabCD device with multiple blood measurements, including HCT has also been presented.[112] Volume ratios of blood plasma and blood cells have also been studied in paper based microfluidics.[183] This simple low cost assay could reveal high, normal or low HCT in half an hour. The Fåhraeus effect has also been used to separate blood cells from plasma and in

combination with optical readout it can reveal the HCT.[80] This system was also further integrated with on-chip ELISA for biomarker detection. In fact, many microfluidic methods capable of blood plasma separation can in combination with optical readout give HCT values. Another approach for measuring HCT has been electrical impedance measurements in blood.[184,185] Electrical impedance is also used in the commercially available POC system i-STAT (Abbott laboratories, Abbott Park, IL, USA) which gives HCT measurements in good agreement with microhaematocrit method.[186] HCT can also be estimated from haemoglobin values and vice versa. This is for example used in the commercially available POC haemoglobin analyser from HemoCue (Radiometer group, Ängelholm, Sweden). Different microfluidic methods for HCT measurement can be preferable depending on the methods for further investigation and biomarker detection in the plasma.

7.2 Acoustic haematocrit measurement

A few acoustic methods for HCT measurements have been presented. Ultrasound has for instance been used to generate a standing wave along the length of a microhaematocrit tube.[187] Several bands of blood cells, one at each pressure node, were formed and the width of them in comparison to the plasma correlated with the HCT. The correlation between speed of sound and HCT has also been used for HCT measurements.[188] Not being entirely acoustic, SAW electrodes in a SAW device have also been used for electrical impedance measurements of blood to reveal HCT.[184] An non-invasive way of measuring HCT in vivo by pulsed ultrasound Doppler measurements has been presented but the performance varied depending on artery used when measuring. [189]

Acoustic haematocrit measurement presented in paper III

The acoustic way of measuring HCT presented in paper III is based on the relative horizontal cross-sectional area between the acoustically packed blood cells and the plasma in a straight channel, Figure 7:1. Unlike the previously presented method where a standing wave was formed along the length in a microhaemtocrit tube, the standing wave in this project was across the width of microfluidic channel. This enables a flow through format where blood cells and plasma eventually can be separated for further biomarker investigation.

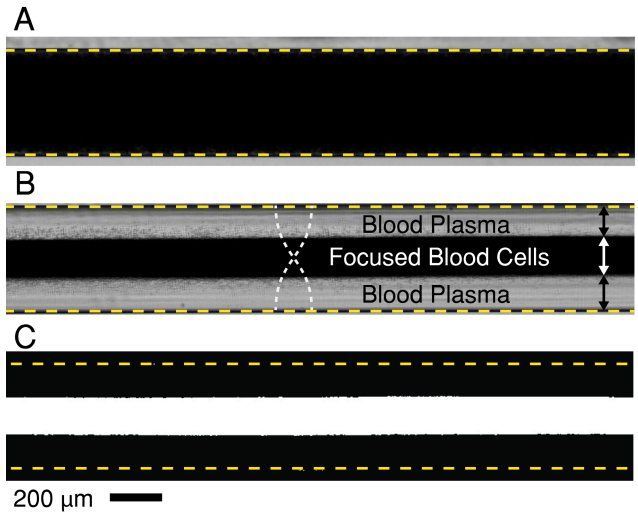


Figure 7:1: A: Channel filled with blood. B: Blood focused to the centre pressure node, 20 s after actuation. C: Inverted binary image used to calculate acoustically packed cell area. Dotted lines shows the channel walls. Reprinted with permission from reference [190], (paper III). Copyright (2017) Elsevier.

Blood was drawn into the microfluidic glass channel and the flow was stopped. When the transducer was actuated, blood cells were focused to the centre while a CCD camera monitored the process. Images from 20 s after actuation were collected and converted to binary black and white images by greyscale thresholding in Matlab (R2016a, MathWorks) before the amount of white pixels were compared with the total number of pixels, Figure 7:1. The calculated acoustically packed cell area was shown to have a linear relationship to HCT measured by a microhaematocrit centrifuge.

As speed of sound depends on the HCT, different HCT would have different optimal working frequencies so to avoid manual tuning of the frequency a frequency sweep was used. This also reduced the presence of acoustic hotspots that can appear when resonances along the channel are found.

7.3 Towards integration and POC system

Acoustic HCT measurement would be possible to integrate into another device which already utilises the acoustic separation of blood and plasma, as in paper I and II. With simple imaging and image analysis HCT can be obtained almost for free in a system that initially separated blood and plasma. These chips however need to be in glass to enable light transmission. Glass, just like silicon, has good reflecting properties and works great with acoustics. Another approach could be to develop a POC device solely for HCT measurements. In that case the setup might be simplified to a glass channel that could be filled by capillary forces and placed in a handheld device that actuates the standing wave, collects images and performs the simple image analysis.

8. Fabrication of an acoustofluidic device

The acoustofluidic devices in paper I, II and IV where all anisotropically wet etched in silicon in-house, while the glass device in paper III was a manufactured by Micronit Microfluidics (Enschede, Netherlands) by isotropic wet etching. In anisotropic etching some directions in the silicon crystal etches faster than others. The orientation of the mask in relation to the crystal planes therefore affects the etch profiles. By turning the structures 45° in relationship to the primary flat on the 100-wafer vertical side walls can be fabricated, working as good reflectors in an acoustofluidic device. Glass does not have crystal planes and the etch rate is therefore the same in all directions, which is called isotropic etching. This results in rounded channels, but they still work well for acoustic focusing in half wavelength resonators as the pressure node in the channel centre display vertical profile much similar to what is seen in a channel with rectangular cross-section as reported by Grenvall *et al.*[191] The procedure for the microfabrication of the acoustofluidic silicon devices in paper I, II and IV are described in the following.

Mask

The channel pattern is drawn on a computer and then printed to a positive photoresist (a polymer which becomes more soluble by light exposure) using a UV-laser on a chromium-covered glass slide. The positive photoresist exposed to the UV-laser is removed in a development step to uncover the chromium layer. Finally, the exposed chromium is etched to reveal the glass surface whereby the structure on the CAD drawing is transferred to the mask as openings in the chromium layer.

Oxidation

As a first step, an 8 h thermal oxidation produces as silicon dioxide layer on top of the silicon wafer. The silicon wafer will spontaneously produce a thin oxide layer when reacting with oxygen, but to create a thicker layer (1 µm), thermal oxidation is performed at high temperature (1000°C) in combination with oxygen or water vapour.

Photolithography

The oxidised silicon wafer is spin coated with a layer of positive photoresist and baked (80°C in 25 min) to reduce the stickiness of the photoresist. The wafer is then exposed to UV-light through the mask to transfer the structures to the photoresist. A developing step reveals the oxide in the UV-exposed structures and an additional baking step (120°C in 30 min) finally hardens the developed photoresist pattern.

Etching

The silicon dioxide revealed in the photoresist can be etched by hydrofluoric acid (HF) and stops as it reaches the silicon surface. When the structures have been transferred from the photoresist to the silicon dioxide the remaining photoresist is removed. The silicon is then etched by potassium hydroxide (KOH) at 80°C to transfer the structures from the oxide into the silicon. All the steps from oxidation to removal of oxide after silicon etch are illustrated in Figure 8:1.

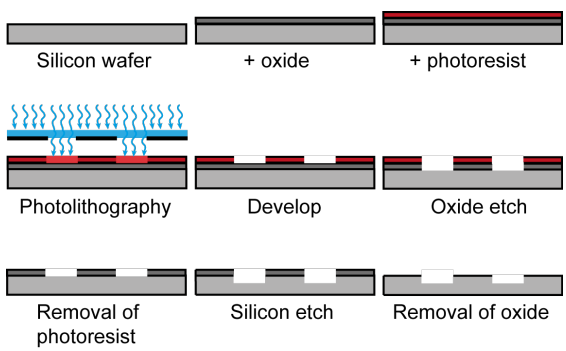


Figure 8:1: Procedure steps to transfer structures from a mask (blue) to photoresist, oxide layer and finally the silicon wafer.

Backside

To fabricate the inlets and outlets to the channel, a second round of oxide patterning and silicon etching is performed. Holes can alternatively be drilled in the silicon or glass.

Bonding

Several chips are often produced from the same wafer and before bonding the wafer is diced into chips and the remaining oxide is removed by HF. A borosilicate glass lid is then anodically bonded to the silicon chip by placing them in contact on a hot plate (500°C) and between two electrodes (1000 V). This will create a charge displacement between the silicon and glass leading to bonding of the two surfaces.

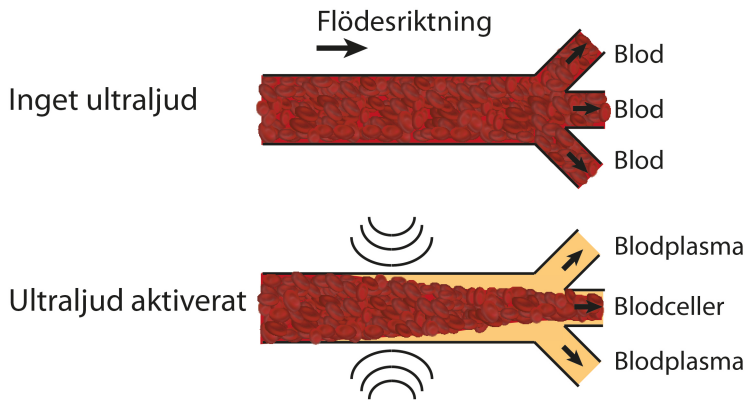
Populärvetenskaplig sammanfattning

Blodprover innehåller stora mängder av information som kan vara till hjälp inom sjukvården, dels för att ställa diagnoser eller för att se hur en behandling går. Markörerna som reflekterar patientens tillstånd brukar kallas biomarkörer och kan vara både fysiska som högt blodtryck och feber eller så kan de vara biologiska föreningar som prostata specifikt antigen (PSA) som ökar vid prostatacancer och blodsocker som kan vara förhöjt hos en diabetespatient. De biologiska biomarkörerna finns i varierande koncentration i blodet, som t.ex. blodsocker som finns i så hög koncentration att det kan mätas direkt i ett blodprov vilket diabetespatienter gör dagligen. Vissa biomarkörer finns i så låg koncentration att de kan vara svåra att hitta på grund av att blodprovet även innehåller flera miljarder blodceller. Om så är fallet brukar man börja med att ta bort blodcellerna med hjälp av en centrifug.

Arbetet i min doktorsavhandling har gått ut på att använda en metod som kallas akustofores för att göra separationen av blodceller och blodplasma istället för att använda en centrifug. Det långsiktiga målet är att utveckla ett mätinstrument som automatiskt kan göra alla nödvändiga steg som blodseparation och detektion av biomarkören i mikroskopiska mikrofluidik-kanaler. När man på detta sätt krymper ner flera olika steg och analyser som normalt sker separat i ett laboratorium till ett enda litet system brukar man kalla dessa för Lab-on-a-chip. Förhoppningen är att analyserna ska ge pålitligare resultat eftersom automatiseringen minskar påverkan av den

mänskliga faktorn. Tanken är också att analyserna ska gå snabbare så att eventuell behandling kan starta tidigare.

Akustofores betyder förflyttining med hjälp av ljud och i mitt fall är det blodceller som jag har flyttat på för att separera dem från blodplasman de normalt befinner sig i. Jag använde ultraljud med högre frekvenser än vad det mänskliga örat kan uppfatta för att flytta cellerna i 1/3 mm breda mikrofluidikkanaler. När ljudet studsar mellan väggarna uppstår en stående våg och blodcellerna flyttar sig mot mitten av kanalen där det bildas en trycknod (figur 11:1). I blodplasman som separeras ut kan man sedan hitta biomarkörer av olika slag. Genom att bygga ihop akustoforesen med detektion av t.ex PSA så kan man få ett mer kompakt system som kan mäta PSA i små blodvolymer och därmed hjälpa till vid diagnostik av prostatacancer (artikel 1).



Figur 11:1. I den övre bilden flödar blod från vänster till höger genom en mikrofluidik-kanal. I slutet delar kanalen upp sig i tre olika delar, och blod flödar ut genom alla tre. I den nedre bilden flödar blod från vänster till höger genom en mikrofluidik-kanal med ultraljud aktiverat. En stående våg skapar en trycknod längs med mitten av kanalen och blodcellerna flyttar sig mot den. I slutet delar sig kanalen och blodcellerna som har flyttats till mitten av kanalen lämnar genom mittutloppet medan blodplasma lämnar genom de två sidoutloppen. Blodplasman kan sedan ledas vidare för önskad analys.

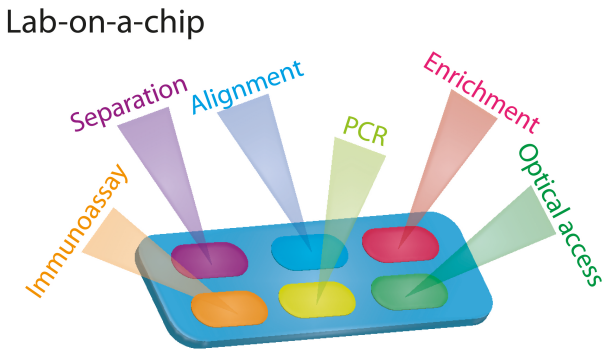
Ett annat tillfälle där ett snabbare detektionssystem skulle göra stor skillnad är när man fått bakterier i blodet, vilket kan leda till sepsis. Sepsis orsakas oftast av en bakterieinfektion och dessa bakterier brukar man leta efter i blodomloppet. Det är livsavgörande att snabbt få reda på vilken sorts bakterie som orsakar sepsis för att kunna behandla patienten med rätt antibiotika som biter på just den bakterien. Eftersom det

är så få bakterier i ett blodprov jämfört med antalet blodceller så brukar man odla bakterierna i ett värmeskåp tills de får så hög koncentration att de kan identifieras. Blododlingen kan ta allt från någon timme upp till flera dagar innan bakterierna blir tillräckligt många för att kunna hittas. Eftersom tidig behandling är livsavgörande så får patienten ofta bredspektrumantibiotika som slår mot en mängd olika bakterier under tiden. Detta är dock något man helst vill undvika eftersom det bidrar till utvecklingen antibiotikaresistenta bakterier. Därför har jag tillsammans med mina kollegor utvecklat ett Lab-on-a-chip-system som först tar bort blodcellerna från plasman med hjälp av akustofores för att sedan fånga in bakterierna med hjälp av ultraljud. I separationssteget så flyttas bara blodcellerna och inte bakterierna mot mitten då bakterierna är för små för att påverkas av ljudet. I den andra delen där koncentreringen sker finns ett litet lokalt ultraljudsfält där bakterierna kan fångas upp och hållas kvar. När bakterierna har koncentrerats tillräckligt så kan ljudet slås av och bakterierna fortsätta vidare till ett litet plastchip som sätts in i en maskin som kan undersöka bakteriernas DNA och på så sätt identifiera dem. Det här systemet har testats på kliniska prover från patienter där blodförgiftning misstänks och resultaten visar att i vissa fall, förmodligen de där bakteriekoncentrationerna från början är lite högre, kan det användas för att identifiera bakterierna på mindre än två timmar (artikel 2). Det är betydligt snabbare än dagens metoder som kan ta från ett upp till ett par dygn. För att även kunna identifiera bakterierna när koncentrationen är lägre utvecklade vi ett förbättrat separationssystemet där vi inte förlorade lika många bakterier som i den första versionen (artikel 4).

Under arbetets gång har jag också upptäckt att genom att bara fotografera kanalen under akustofores av blod så kan man läsa ut blodprovets hematokrit, dvs. volymprocenten blodceller i blodprovet. Hematokritvärdet kan användas som en biomarkör då både ovanligt låga och ovanligt höga cellkoncentrationer är tecken på sjukdom. Arean som blodcellerna upptar när de flyttats till mitten av kanalen i förhållande till kanalbredden stämmer väl med hematokrit mätt på standardvis (artikel 3).

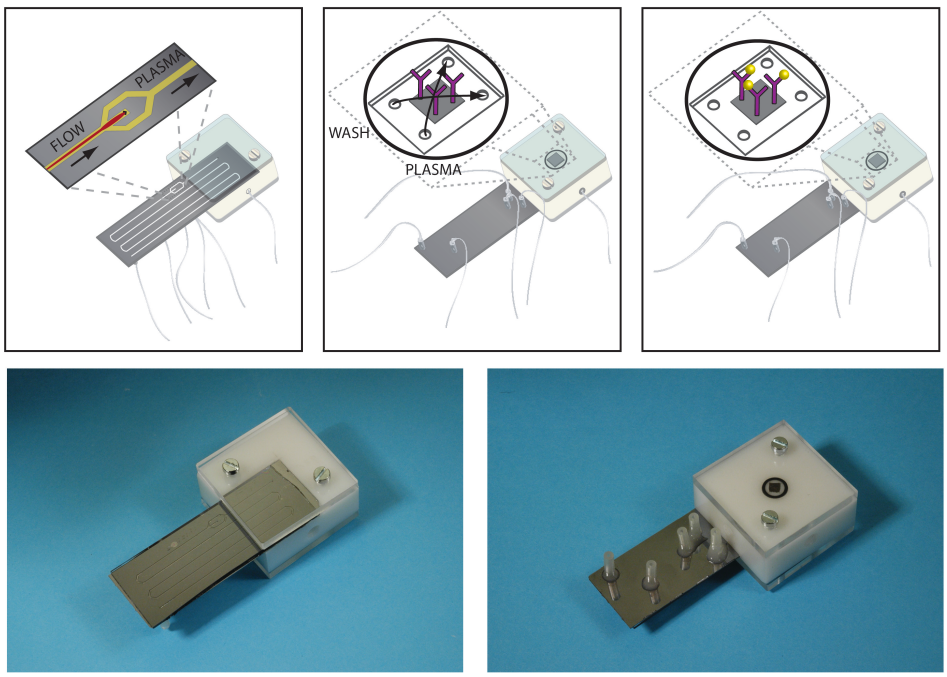
Summary of papers

All four papers included in this thesis touch upon clinical blood based applications of acoustophoresis. The overall goal has been to move towards microfluidic sample preparation, such as separation and enrichment, which are important building blocks in the development of Lab-on-a-chip devices. Acoustofluidic blood plasma separation has proven to be possible to integrate with further steps such as acoustic enrichment, immunoassays and PCR. The possibility to use glass chips also opens up for optical investigation of the produced plasma.



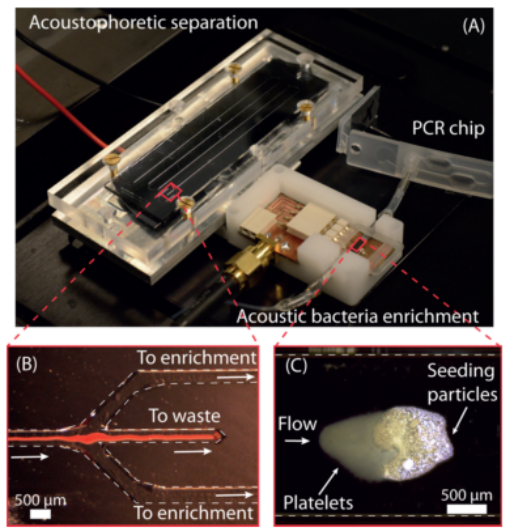
Integrated acoustic immunoaffinity-capture (IAI) platform for detection of PSA from whole blood samples

Integration of acoustophoresis based blood plasma separation with a miniaturised immunoassay made it possible to detect PSA direct from undiluted whole blood. Whole blood spiked with PSA entered the separation chip (50 µL/min) and blood cells were acoustically focused to the centre and sequentially removed while plasma (10 µL/min) continued to flow over a porous silicon chip spotted with capturing antibodies. PSA was captured and the plasma was washed away before the porous silicon chip was removed and incubated with detection antibodies followed by fluorescence detection. Clinically useful plasma was produced at a yield of 20% of the total blood volume which enabled detection of PSA in relevant concentrations (1.7-100 ng/mL). The plasma incubation time including wash was reduced from earlier reports of 75 min to 20 min due to the convective transport of fresh plasma across the capturing site.



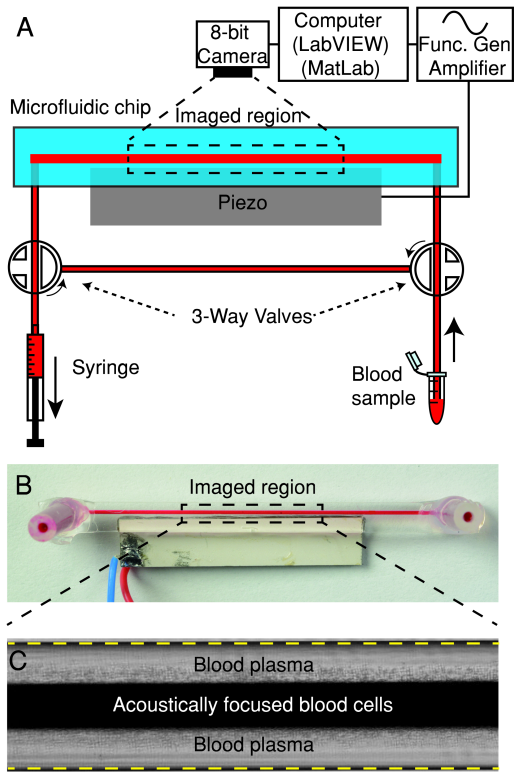
Integrated acoustic separation, enrichment and microchip polymerase chain reaction detection of bacteria from blood for rapid sepsis diagnostics

By integration of acoustic blood separation, acoustic trapping for bacteria enrichment and microchip PCR (A) enabled rapid (<2 h) identification of bacteria from both spiked blood samples and sepsis patient blood samples. The slightly diluted blood sample (70% whole blood) was processed in the acoustophoretic separation chip where RBCs were removed sequentially through outlets in the centre of the channel bottom while a cell free plasma was generated along the channel sides. At a trifurcation at the end of the channel (B) the remaining blood cells were removed while bacteria suspended in plasma proceeded to the trapping capillary. The trapping site was prefilled with larger seeding particles to enable capturing of the bacteria by acoustic secondary radiation forces as bacteria are too small to be trapped by themselves (C). The trapped bacteria were washed and released into a PCR microchip with dried reagents, which in turn was placed in a PCR thermoscycling instrument for bacteria identification. The whole process, from whole blood to identification was completed in less than 2 h which is considerably faster than conventional detection methods. The integrated system was able to detect *E. coli* in the clinical samples that displayed an early alarm in the corresponding blood culture, indicating that the system can detect bacteria in the upper part of relevant bacteria concentrations.



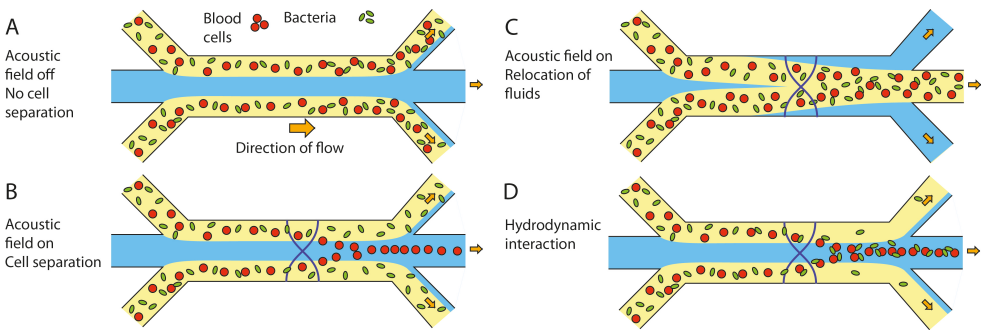
Acoustofluidic hematocrit determination

This paper presents an acoustofluidic method for measuring haematocrit (HCT) in blood samples. Blood cells were focused to the centreline of the channel while imaged and the relative image areas of the focused blood cells and the plasma regions were calculated by converting the image to binary black and white via greyscale thresholding. This acoustically packed cell area was shown to have a linear relationship to HCT measured by the centrifugation-based reference method. This setup generated a readout in only 2 s with an accuracy of approximately 3% points of HCT. By extending readout to 20 s, the error was reduced to 1% point. This way of measuring HCT could be integrated in-line with sequential blood based acoustophoretic applications.



Acoustic impedance matched buffers enable separation of bacteria from blood cells at high cell concentrations

Blood cells and bacteria were acoustically separated to enable a faster bacteria identification. Blood was laminated along the side walls by a buffer entering the centre (A). A half wavelength acoustic resonance across the width of the channel focused the blood cells into the buffer stream (blue, B). To avoid focusing of the entire sample, so called media switching (C), density medium was mixed into the centre buffer to reach an acoustic impedance (density times speed of sound) higher than for the blood sample used. Diluted blood samples were needed to avoid that the blood cells hydrodynamically transport plasma and bacteria in to the centre buffer (D). The separation of bacteria from blood cells displayed high bacteria recovery while removing most of the blood cells in a reasonable time, making it a promising first step in a device for fast bacteria identification.



Acknowledgements

The last year of my master's studies in Linköping came to an end and I wondered what I should do in the future. We had a guest lecturer visiting from Lund and he talked about microfluidics and how to magically move around blood in channels using ultrasound. The inspiring lecture and the interesting project made me think about the possibility to do my master's thesis in Lund. In combination with the often stated claim ''*It never rains in Skåne*'' from Patrik, now my husband, I decided to contact the lecturer, Thomas Laurell, to investigate the possibility of doing a master's thesis work in his research group. Suddenly, I had not only finished my master's thesis but also started my PhD studies.

To begin with, I would like to express my gratitude towards my supervisor Professor Thomas Laurell for introducing me to the field during the lecture in Linköping as well as guiding me through my PhD studies. Your optimistic mind and your visons have been of great importance, especially when not everything goes as planned. Thanks to my assistant supervisor Johan Malm for the clinical perspective as well as the very last minute proofreading of my thesis. Thanks to my assistant supervisor Pelle Ohlsson for doing everything very thoroughly, always having a specific scientific paper as answer to my questions and for being optimistic about everything. Johan Nilsson, thanks for being a great chief and making me feel welcome to the family of Elmät.

Thanks to all my former and current colleagues at Elmät/BME. Belinda Lyddby for welcoming me into the group when I first came to Lund. Maria Antfolk for learning the basics about acoustophoresis together with me and for being the best conference

company. Asilah Ahmad Tajudin for great office company and for introducing me to the PSA work. Mikael Evander for being a morning person, for all the fun lab hours and for making us celebrate at least a few achievements during our collaboration. Ola Jakobsson for effective collaboration and for your constant pranks. Hong Yan for being tiptop office company. The Sol-group for the best meetings. Carl Grenvall for fun conferences. Björn Hammarström for letting me steal your lab setup when you left. Per Augustsson for always being very helpful and good at explaining. Andreas Lenshof for letting me take over the bloody work and for the wonderful sound of coffee break when you stir with your spoon in the coffee coup. Linus Jonsson for helping me with fabrication when I was too pregnant to fit into the clean room suit. Axel Tojo for help with everything from coffee soaked laptop to milling holders and exporting to the correct type of pdf. Anna Fornell for swimming company at 5 am. Eva Undvall and Anke Urbansky for great office company, Tradera updates and scientific input. Cecilia Magnusson and Franzi Olm for nice lab spirit and interesting discussions. Mathias Ohlin for bringing fresh ideas into the group. Carl Johannesson for last minute equation check. Désirée Jarebrant, Ulrika Westerdahl, Ammi Lindgren and Malgo Luczak for all help with administration, without you everything would have been so much harder. All Elmät/BME people for fun crayfish parties, AWs and working hours.

Thanks to all my supportive friends and family. My dear friends Elin, Linda, Charlotte and Sara for inspiring work discussions while visiting different parts of the world. My dear family for being who you are. My brother for showing how things should be done. My new family for all help with Ellen.

Last but not least, thanks to my husband Patrik and my daughter Ellen who mean everything to me. If Patrik had not convinced me that Skåne was the place to live, I would probably never have written this doctoral thesis.

REFERENCES

1 Manz, A., Graber, N. & Widmer, H. M. Miniaturized Total Chemical Analysis System: a Novel Concept for Chemical Sensing. *Sensors and Actuators B: Chemical* **1**, 244-248 (1990).

2 Tortora, G. J. & Grabowski, S. R. *Principles of Anatomy and Physiology*. 8th edn, Vol. 8 th 553-577 (Biological Sciences Textbooks, Inc. , 1996).

3 Cushing, K. W. *et al.* Ultrasound Characterization of Microbead and Cell Suspensions by Speed of Sound Measurements of Neutrally Buoyant Samples. *Anal Chem* **89**, 8917-8923, doi:10.1021/acs.analchem.7b01388 (2017).

4 Sethu, P., Sin, A. & Toner, M. Microfluidic diffusive filter for apheresis (leukapheresis). *Lab Chip* **6**, 83-89, doi:10.1039/b512049g (2006).

5 Hanash, S. M., Pitteri, S. J. & Faca, V. M. Mining the plasma proteome for cancer biomarkers. *Nature* **452**, 571-579, doi:10.1038/nature06916 (2008).

6 Anderson, N. L. & Anderson, N. G. The Human Plasma Proteome. *Molecular & Cellular Proteomics* **1**, 845-867, doi:10.1074/mcp.R200007-MCP200 (2002).

7 Anderson, L. Six decades searching for meaning in the proteome. *J Proteomics* **107**, 24-30, doi:10.1016/j.jprot.2014.03.005 (2014).

8 Polanski, M. & Anderson, L. A list of candidate cancer biomarkers for targeted proteomics. *Biomarker Insights* **1**, 1-48 (2006).

9 Clyne, B. & Olshaker, J. S. The C-reactive protein *The Journal of Emergency Medicine* **17**, 1019-1025 (1999).

10 Tsalik, E. L. *et al.* Discriminative value of inflammatory biomarkers for suspected sepsis. *J Emerg Med* **43**, 97-106, doi:10.1016/j.jemermed.2011.05.072 (2012).
11 Anderson, N. L. The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. *Clin Chem* **56**, 177-185, doi:10.1373/clinchem.2009.126706 (2010).
12 Psychogios, N. *et al.* The Human Serum Metabolome. *PLoS One* **6**, 1-23, doi:10.1371/journal (2011).
13 Kanji, S. *et al.* Reliability of point-of-care testing for glucose measurement in critically ill adults*. *Critical Care Medicine* **33**, 2778-2785, doi:10.1097/01.ccm.0000189939.10881.60 (2005).
14 Yin, P., Lehmann, R. & Xu, G. Effects of pre-analytical processes on blood samples used in metabolomics studies. *Anal Bioanal Chem* **407**, 4879-4892, doi:10.1007/s00216-015-8565-x (2015).
15 Wagner, J. Free DNA - new potential in clinical laboratory diagnostics? *Biochemia Medica* **22**, 24-38 (2012).
16 Schwarzenbach, H., Hoon, D. S. & Pantel, K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* **11**, 426-437, doi:10.1038/nrc3066 (2011).
17 Kubaczkova, V., Vrabel, D., Sedlarikova, L., Besse, L. & Sevcikova, S. Cell-free DNA - Minimally invasive marker of hematological malignancies. *Eur J Haematol* **99**, 291-299, doi:10.1111/ejh.12925 (2017).
18 Wan, J. C. M. *et al.* Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* **17**, 223-238, doi:10.1038/nrc.2017.7 (2017).
19 Rainer, T. H. *et al.* Prognostic use of circulating plasma nucleic acid concentrations in patients with acute stroke. *Clin Chem* **49**, 562-569 (2003).
20 Franklin, B. S. *et al.* Plasma circulating nucleic acids levels increase according to the morbidity of Plasmodium vivax malaria. *PLoS One* **6**, e19842, doi:10.1371/journal.pone.0019842 (2011).
21 Wittek, M., Stürmer, M., Doerr, H. W. & Berger, A. Molecular assays for monitoring HIV infection and antiretroviral therapy. *Expert Review of Molecular Diagnostics* 7, 237-246, doi:10.1586/14737159.7.3.237 (2007).
22 Boukouris, S. & Mathivanan, S. Exosomes in bodily fluids are a highly stable resource of disease biomarkers. *Proteomics Clin Appl* **9**, 358-367, doi:10.1002/prca.201400114 (2015).
23 Schiro, A. *et al.* Endothelial microparticles as conveyors of information in atherosclerotic disease. *Atherosclerosis* **234**, 295-302, doi:10.1016/j.atherosclerosis.2014.03.019 (2014).
24 Loyer, X., Vion, A. C., Tedgui, A. & Boulanger, C. M. Microvesicles as cell-cell messengers in cardiovascular diseases. *Circ Res* **114**, 345-353, doi:10.1161/CIRCRESAHA.113.300858 (2014).
25 Graham, M. The Coulter principle: foundation of an industry. *Journal of the Association for Laboratory Automation* **8**, 72-81, doi:10.1016/s1535-5535(03)00023-6 (2003).

26 Brown, M. & Wittwer, C. Flow cytometry: Principles and clinical applications in Hematology. *Clin Chem* **46**, 1221-1229 (2000).
27 Recommended method for the determination of packed cell volume by centrifugation - World Health Organization (WHO). (2000).
28 Lehmann, L. E. *et al.* Improved detection of blood stream pathogens by real-time PCR in severe sepsis. *Intensive Care Med* **36**, 49-56, doi:10.1007/s00134-009-1608-z (2010).
29 Klose, J. Protein Mapping by Combined Isoelectric Focusing and Electrophoresis of Mouse Tissues. *Humangenetik* **26**, 231-234 (1975).
30 O´Farrell, P. H. High resolution two-dimensional electrophoresis of proteins. *The Journal of Biological Chemistry* **250**, 4007-4021 (1975).
31 Parker, C. E., Pearson, T. W., Anderson, N. L. & Borchers, C. H. Mass-sepctrometry-based clinical proteomics - a review and prospective. *Analyst* **135**, 1830-1838 (2010).
32 Lequin, R. M. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin Chem* **51**, 2415-2418, doi:10.1373/clinchem.2005.051532 (2005).
33 Engvall, E. & Perlmann, P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871-874 (1971).
34 Nguyen, N.-T. & Wereley, S. T. *Fundamentals and applications of microfluidics*. (Artech House, Inc., 2002).
35 Kundt, A. & Lehmann, O. Longitudinal vibrations and acoustic figures in cylindrical columns of liquid. *Annalen der Physik und Chemie* **153**, 1 (1874).
36 King, L. V. <34 acoustic radiation force King.pdf>. *Proceedings of the Royal Society of London* **147**, 212-240 (1934).
37 Yosioka, K. & Kawasima, Y. Acoustic radiation pressure on compressible sphere. *Acustica* **5**, 167-173 (1955).
38 Gorkov, L. P. Force acting on a small particle in an acoustic field within an ideal fluid. *Doklady Akademii Nauk Sssr* **140**, 88-91 (19621).
39 Bruus, H. Acoustofluidics 7: The acoustic radiation force on small particles. *Lab Chip* **12**, 1014-1021, doi:10.1039/c2lc21068a (2012).
40 Bjerknes, V. F. K. Die Kraftfelder. *Vieweg und Sohn: Braunschweig, Germany* (1909).
41 Hammarstrom, B., Laurell, T. & Nilsson, J. Seed particle-enabled acoustic trapping of bacteria and nanoparticles in continuous flow systems. *Lab Chip* **12**, 4296-4304, doi:10.1039/c2lc40697g (2012).
42 Gröschl, M.; Ultrasonic Separation of Suspended Particles - Part 1, *Acoustica* **84**, 432-447 (1998)
43 Weiser, M. A. H., Apfel, R. E. Interparticle forces on red cells in a standing wave field. *Acustica* **54**, 114-119 (1984)
44 Wiklund, M., Green, R. & Ohlin, M. Acoustofluidics 14: Applications of acoustic streaming in microfluidic devices. *Lab Chip* **12**, 2438-2451, doi:10.1039/c2lc40203c (2012).
45 Rayleigh, L. On the circulations of air observed in Kundt's tubes, and some allied acoustical problems. *Phiolos Trans R Soc Lond* **175**, 1-21 (1884).

46 Schlichting, H. Berechnung obener periodischer grenzschichtströmungen. *Physik Z* **33**, 327-335 (1932).
47 Nyborg, W. L. Acoustic Streaming near a Boundary. *The Journal of the Acoustical Society of America* **30**, 329-339, doi:10.1121/1.1909587 (1958).
48 Barnkob, R., Augustsson, P., Laurell, T. & Bruus, H. Acoustic radiation- and streaming-induced microparticle velocities determined by microparticle image velocimetry in an ultrasound symmetry plane. *Phys Rev E Stat Nonlin Soft Matter Phys* **86**, 056307, doi:10.1103/PhysRevE.86.056307 (2012).
49 Muller, P. B., Barnkob, R., Jensen, M. J. H. & Bruus, H. A numerical study of microparticle acoustophoresis driven by acoustic radiation forces and streaming-induced drag forces. *Lab Chip* **12**, 4617-4627 (2012).
50 Lenshof, A., Evander, M., Laurell, T. & Nilsson, J. Acoustofluidics 5: Building microfluidic acoustic resonators. *Lab Chip* **12**, 684-695, doi:10.1039/c1lc20996e (2012).
51 Augustsson, P., Magnusson, C., Nordin, M., Lilja, H. & Laurell, T. Microfluidic, label-free enrichment of prostate cancer cells in blood based on acoustophoresis. *Anal Chem* **84**, 7954-7962, doi:10.1021/ac301723s (2012).
52 Plebani, M. Exploring the iceberg of errors in laboratory medicine. *Clin Chim Acta* **404**, 16-23, doi:10.1016/j.cca.2009.03.022 (2009).
53 Mariella, R., Jr. Sample preparation: the weak link in microfluidics-based biodetection. *Biomed Microdevices* **10**, 777, doi:10.1007/s10544-008-9190-7 (2008).
54 Wilding, P., Pfahler, J., Bau, H. H., Zemel, J. N. & Kricka, L. J. Manipulation and flow of biologial fluids in straight channels micromachined in silicon. *Clin Chem* **40**, 43-47 (1994).
55 Thorslund, S., Klett, O., Nikolajeff, F., Markides, K. & Bergquist, J. A hybrid poly(dimethylsiloxane) microsystem for on-chip whole blood filtration optimized for steroid screening. *Biomed Microdevices* **8**, 73-79, doi:10.1007/s10544-006-6385-7 (2006).
56 Wang, S. *et al.* Simple filter microchip for rapid separation of plasma and viruses from whole blood. *Int J Nanomedicine* **7**, 5019-5028, doi:10.2147/IJN.S32579 (2012).
57 Gong, M. M., Macdonald, B. D., Vu Nguyen, T., Van Nguyen, K. & Sinton, D. Field tested milliliter-scale blood filtration device for point-of-care applications. *Biomicrofluidics* 7, 44111, doi:10.1063/1.4817792 (2013).
58 Moorthy, J. & Beebe, D. J. In situ fabricated porous filters for microsystems. *Lab Chip* **3**, 62-66, doi:10.1039/b300450c (2003).
59 Li, C., Liu, C., Xu, Z. & Li, J. Extraction of plasma from whole blood using a deposited microbead plug (DMBP) in a capillary-driven microfluidic device. *Biomed Microdevices* **14**, 565-572, doi:10.1007/s10544-012-9635-x (2012).
60 Shim, J. S. & Ahn, C. H. An on-chip whole blood/plasma separator using hetero-packed beads at the inlet of a microchannel. *Lab Chip* **12**, 863-866, doi:10.1039/c2lc21009f (2012).

61 Li, C., Liu, C., Xu, Z. & Li, J. A power-free deposited microbead plug-based microfluidic chip for whole-blood immunoassay. *Microfluidics and Nanofluidics* **12**, 829-834, doi:10.1007/s10404-011-0911-5 (2011).
62 Lee, K. K. & Ahn, C. H. A new on-chip whole blood/plasma separator driven by asymmetric capillary forces. *Lab Chip* **13**, 3261-3267, doi:10.1039/c3lc50370d (2013).
63 Aran, K. *et al.* Microfiltration platform for continuous blood plasma protein extraction from whole blood during cardiac surgery. *Lab Chip* **11**, 2858-2868, doi:10.1039/c1lc20080a (2011).
64 Chen, P. C., Chen, C. C. & Young, K. C. Characterization of thermoplastic microfiltration chip for the separation of blood plasma from human blood. *Biomicrofluidics* **10**, 054112, doi:10.1063/1.4964388 (2016).
65 Crowley, T. A. & Pizziconi, V. Isolation of plasma from whole blood using planar microfilters for lab-on-a-chip applications. *Lab Chip* **5**, 922-929, doi:10.1039/b502930a (2005).
66 VanDelinder, V. & Groisman, A. Separation of Plasma from Whole Human Blood in a Continuous Cross-Flow in a Molded Microfluidic Device. *Anal Chem* **78**, 3765-3771 (2006).
67 Madadi, H., Casals-Terre, J. & Mohammadi, M. Self-driven filter-based blood plasma separator microfluidic chip for point-of-care testing. *Biofabrication* **7**, 025007, doi:10.1088/1758-5090/7/2/025007 (2015).
68 Aota, A. *et al.* Microchip-based plasma separation from whole blood via axial migration of blood cells. *Analytical Sciences* **27**, 1173-1178 (2011).
69 Son, J. H. *et al.* Hemolysis-free blood plasma separation. *Lab Chip* **14**, 2287-2292, doi:10.1039/c4lc00149d (2014).
70 Tachi, T., Kaji, N., Tokeshi, M. & Baba, Y. Simultaneous Separation, Metering, and Dilution of Plasma from Human Whole Blood in a Microfluidic System *Anal Chem* **81**, 3194-3198 (2009).
71 Maria, M. S., Rakesh, P. E., Chandra, T. S. & Sen, A. K. Capillary flow-driven microfluidic device with wettability gradient and sedimentation effects for blood plasma separation. *Sci Rep* **7**, 43457, doi:10.1038/srep43457 (2017).
72 Liu, C. *et al.* Membrane-based, sedimentation-assisted plasma separator for point-of-care applications. *Anal Chem* **85**, 10463-10470, doi:10.1021/ac402459h (2013).
73 Dimov, I. K. *et al.* Stand-alone self-powered integrated microfluidic blood analysis system (SIMBAS). *Lab Chip* **11**, 845-850, doi:10.1039/c0lc00403k (2011).
74 Wu, C.-C. Blood Cell-free Plasma Separated from Blood Samples with a Cascading Weir-type Microfilter Using Dead-end Filtration. *Journal of Medical and Biological Engineering* **32**, doi:10.5405/jmbe.937 (2012).
75 Zhang, X. B. *et al.* Gravitational sedimentation induced blood delamination for continuous plasma separation on a microfluidics chip. *Anal Chem* **84**, 3780-3786, doi:10.1021/ac3003616 (2012).
76 Fåhraeus, R. The suspension stability of the blood. *Physiological reviews* **9**, 241-274 (1929).

77 Fåhraeus, R. & Lindqvist, T. The viscosity of the blood in narrow capillary tubes. *Am J Physiol. Lefacy content* **96**, 562-568 (1930).
78 Fung, J.-C. Stochastic flow in capillary blood vessels. *microvascular research* **5**, 34-48 (1973).
79 Faivre, M., Abkarian, M., Bickraj, K. & Stone, H. A. Geometrical focusing of cells in a microfluidic device: An approach to separate blood plasma. *Biorheology* **43**, 147-159 (2006).
80 Browne, A. W., Ramasamy, L., Cripe, T. P. & Ahn, C. H. A lab-on-a-chip for rapid blood separation and quantification of hematocrit and serum analytes. *Lab Chip* **11**, 2440-2446, doi:10.1039/c1lc20144a (2011).
81 Yang, S., Undar, A. & Zahn, J. D. A microfluidic device for continuous, real time blood plasma separation. *Lab Chip* **6**, 871-880, doi:10.1039/b516401j (2006).
82 Jäggi, R. D., Sandoz, R. & Effenhauser, C. S. Microfluidic depletion of red blood cells from whole blood in high-aspect-ratio microchannels. *Microfluidics and Nanofluidics* **3**, 47-53, doi:10.1007/s10404-006-0104-9 (2006).
83 Fan, R. *et al.* Integrated barcode chips for rapid, multiplexed analysis of proteins in microliter quantities of blood. *Nat Biotechnol* **26**, 1373-1378, doi:10.1038/nbt.1507 (2008).
84 Di Carlo, D., Edd, J. F., Irimia, D., Tompkins, R. D. & Toner, M. Equilibrium Separtion and Filtration of Particles Using Differential Inertial Focusing. *Anal Chem* **80**, 2204-2211 (2008).
85 Sollier, E., Cubizolles, M., Fouillet, Y. & Achard, J. L. Fast and continuous plasma extraction from whole human blood based on expanding cell-free layer devices. *Biomed Microdevices* **12**, 485-497, doi:10.1007/s10544-010-9405-6 (2010).
86 Marchalot, J., Fouillet, Y. & Achard, J.-L. Multi-step microfluidic system for blood plasma separation: architecture and separation efficiency. *Microfluidics and Nanofluidics* **17**, 167-180, doi:10.1007/s10404-013-1296-4 (2013).
87 Kersaudy-Kerhoas, M., Dhariwal, R., Desmulliez, M. P. Y. & Jouvet, L. Hydrodynamic blood plasma separation in microfluidic channels. *Microfluidics and Nanofluidics* **8**, 105-114, doi:10.1007/s10404-009-0450-5 (2009).
88 Kersaudy-Kerhoas, M., Kavanagh, D. M., Dhariwal, R. S., Campbell, C. J. & Desmulliez, M. P. Validation of a blood plasma separation system by biomarker detection. *Lab Chip* **10**, 1587-1595, doi:10.1039/b926834k (2010).
89 Rodriguez-Villarreal, A. I., Arundell, M., Carmona, M. & Samitier, J. High flow rate microfluidic device for blood plasma separation using a range of temperatures. *Lab Chip* **10**, 211-219, doi:10.1039/b904531g (2010).
90 Blatter, C., Jurischka, R., Schoth, A., Kerth, P. & Menz, W. in *Separation of blood cells and plasma in microchannel bend structures, SPIE Lab-on-a-Chip: Platforms, Devices, and Applications, 2004, Philadelphia, Pennsylvania, USA.*
91 Blatter, C. *et al.* in *Separation of blood on microchannel Bends, Annual International Conference of the IEEE EMBS, 2004, San Francisco, CA, USA.*

92 Tripathi, S., Prabhakar, A., Kumar, N., Singh, S. G. & Agrawal, A. Blood plasma separation in elevated dimension T-shaped microchannel. *Biomed Microdevices* **15**, 415-425, doi:10.1007/s10544-013-9738-z (2013).

93 Prabhakar, A., Kumar, Y. V. B. V., Tripathi, S. & Agrawal, A. A novel, compact and efficient microchannel arrangement with multiple hydrodynamic effects for blood plasma separation. *Microfluidics and Nanofluidics* **18**, 995-1006, doi:10.1007/s10404-014-1488-6 (2015).

94 Tripathi, S., Kumar, Y. V., Agrawal, A., Prabhakar, A. & Joshi, S. S. Microdevice for plasma separation from whole human blood using bio-physical and geometrical effects. *Sci Rep* **6**, 26749, doi:10.1038/srep26749 (2016).

95 Di Carlo, D. Inertial microfluidics. *Lab Chip* **9**, 3038-3046, doi:10.1039/b912547g (2009).

96 Mach, A. J. & Di Carlo, D. Continuous scalable blood filtration device using inertial microfluidics. *Biotechnol Bioeng* **107**, 302-311, doi:10.1002/bit.22833 (2010).

97 Gossett, D. R. & di Carlo, D. Particle focusing mechanisms in curving confined flows. *Anal Chem* **81**, 8459-8465 (2009).

98 Nivedita, N. & Papautsky, I. Continuous separation of blood cells in spiral microfluidic devices. *Biomicrofluidics* 7, 54101, doi:10.1063/1.4819275 (2013).

99 Lee, M. G. *et al.* Inertial blood plasma separation in a contraction–expansion array microchannel. *Applied Physics Letters* **98**, doi:10.1063/1.3601745 (2011).

100 Huang, L. R., Cox, E. C., Austin, R. H. & Sturm, J. C. Continuous Particle Separation Through Deterministic Lateral Displacement. *Science* **304**, 987-990 (2004).

101 Davis, J. A. *et al.* Deterministic Hydrodynmics: Taking blood apart. *PNAS* **103**, 14779-14784 (2006).

102 Holm, S. H., Beech, J. P., Barrett, M. P. & Tegenfeldt, J. O. Separation of parasites from human blood using deterministic lateral displacement. *Lab Chip* **11**, 1326-1332, doi:10.1039/c0lc00560f (2011).

103 Beech, J. P., Holm, S. H., Adolfsson, K. & Tegenfeldt, J. O. Sorting cells by size, shape and deformability. *Lab Chip* **12**, 1048-1051, doi:10.1039/c2lc21083e (2012).

104 Zeming, K. K., Ranjan, S. & Zhang, Y. Rotational separation of non-spherical bioparticles using I-shaped pillar arrays in a microfluidic device. *Nat Commun* **4**, 1625, doi:10.1038/ncomms2653 (2013).

105 Zeming, K. K., Salafi, T., Chen, C. H. & Zhang, Y. Asymmetrical Deterministic Lateral Displacement Gaps for Dual Functions of Enhanced Separation and Throughput of Red Blood Cells. *Sci Rep* **6**, 22934, doi:10.1038/srep22934 (2016).

106 Holm, S. H., Beech, J. P., Barrett, M. P. & Tegenfeldt, J. O. Simplifying microfluidic separation devices towards field-detection of blood parasites. *Analytical Methods* **8**, 3291-3300, doi:10.1039/c6ay00443a (2016).

107 Yang, X., Forouzan, O., Brown, T. P. & Shevkoplyas, S. S. Integrated separation of blood plasma from whole blood for microfluidic paper-based analytical devices. *Lab Chip* **12**, 274-280, doi:10.1039/c1lc20803a (2012).

108 Songjaroen, T., Dungchai, W., Chailapakul, O., Henry, C. S. & Laiwattanapaisal, W. Blood separation on microfluidic paper-based analytical devices. *Lab Chip* **12**, 3392-3398, doi:10.1039/c2lc21299d (2012).
109 Vella, S. J. *et al.* Measuring markers of liver function using a micropatterned paper device designed for blood from a fingerstick. *Anal Chem* **84**, 2883-2891, doi:10.1021/ac203434x (2012).
110 Kar, S., Maiti, T. K. & Chakraborty, S. Capillarity-driven blood plasma separation on paper-based devices. *Analyst* **140**, 6473-6476, doi:10.1039/c5an00849b (2015).
111 Noiphung, J. *et al.* Electrochemical detection of glucose from whole blood using paper-based microfluidic devices. *Anal Chim Acta* **788**, 39-45, doi:10.1016/j.aca.2013.06.021 (2013).
112 Schembri, C. T., Ostoich, V., Lingane, P. J., Burd, T. L. & Buhl, S. N. Portable simultaneous multiple analyte whole blood analyzer for point-of-care testing. *Clin Chem* **38**, 1665-1670 (1992).
113 Haeberle, S., Brenner, T., Zengerle, R. & Ducree, J. Centrifugal extraction of plasma from whole blood on a rotating disk. *Lab Chip* **6**, 776-781, doi:10.1039/b604145k (2006).
114 Lee, B. S. *et al.* ChaA fully automated immunoassay from whole blood in a disc. *Lab Chip* **9**, 1548-1555, doi:10.1016/s1043-321x(09)79309-9 (2009).
115 Schaff, U. Y. & Sommer, G. J. Whole blood immunoassay based on centrifugal bead sedimentation. *Clin Chem* **57**, 753-761, doi:10.1373/clinchem.2011.162206 (2011).
116 Amasia, M. & Madou, M. Large-volume centrifugal microfluidic device for blood plasma separation. *Bioanalysis* **2**, 1701-1710 (2010).
117 Gorkin, R. *et al.* Centrifugal microfluidics for biomedical applications. *Lab Chip* **10**, 1758-1773, doi:10.1039/b924109d (2010).
118 Nakashima, Y., Hata, S. & Yasuda, T. Blood plasma separation and extraction from a minute amount of blood using dielectrophoretic and capillary forces. *Sensors and Actuators B: Chemical* **145**, 561-569, doi:10.1016/j.snb.2009.11.070 (2010).
119 Szydzik, C., Khoshmanesh, K., Mitchell, A. & Karnutsch, C. Microfluidic platform for separation and extraction of plasma from whole blood using dielectrophoresis. *Biomicrofluidics* **9**, 064120, doi:10.1063/1.4938391 (2015).
120 Jiang, H., Weng, X., Chon, C. H., Wu, X. & Li, D. A microfluidic chip for blood plasma separation using electro-osmotic flow control. *Journal of Micromechanics and Microengineering* **21**, doi:10.1088/0960-1317/21/8/085019 (2011).
121 Mohammadi, M., Madadi, H., Casals-Terre, J. & Sellares, J. Hydrodynamic and direct-current insulator-based dielectrophoresis (H-DC-iDEP) microfluidic blood plasma separation. *Anal Bioanal Chem* **407**, 4733-4744, doi:10.1007/s00216-015-8678-2 (2015).
122 Petersson, F., Nilsson, A., Holm, C., Jonsson, H. & Laurell, T. Separation of lipids from blood utilizing ultrasonic standing waves in microfluidic channels. *Analyst* **129**, 938-943, doi:10.1039/b409139f (2004).
123 Lenshof, A. *et al.* Acoustic Whole Blood Plasmapheresis Chip for Prostate Specific Antigen Microarray Diagnostics. *Anal Chem* **81**, 6030-6037 (2009).

124 Dykes, J., Lenshof, A., Astrand-Grundstrom, I. B., Laurell, T. & Scheding, S. Efficient removal of platelets from peripheral blood progenitor cell products using a novel micro-chip based acoustophoretic platform. *PLoS One* **6**, e23074, doi:10.1371/journal.pone.0023074 (2011).
125 Tajudin, A. A. *et al.* Integrated acoustic immunoaffinity-capture (IAI) platform for detection of PSA from whole blood samples. *Lab Chip* **13**, 1790-1796, doi:10.1039/c3lc41269e (2013).
126 Lenshof, A. *et al.* Efficient Purification of CD41 Lymphocytes from Peripheral Blood Progenitor Cell Products Using Affinity Bead Acoustophoresis. *Cytometry Part A* **85**, 933-941, doi:10.1002/cyto.22507 (2014).
127 Antfolk, M., Antfolk, C., Lilja, H., Laurell, T. & Augustsson, P. A single inlet two-stage acoustophoresis chip enabling tumor cell enrichment from white blood cells. *Lab Chip* **15**, 2102-2109, doi:10.1039/c5lc00078e (2015).
128 Grenvall, C., Magnusson, C., Lilja, H. & Laurell, T. Concurrent isolation of lymphocytes and granulocytes using prefocused free flow acoustophoresis. *Anal Chem* **87**, 5596-5604, doi:10.1021/acs.analchem.5b00370 (2015).
129 Tenje, M. *et al.* Acoustophoretic removal of proteins from blood components. *Biomed Microdevices* **17**, 95, doi:10.1007/s10544-015-0003-5 (2015).
130 Antfolk, M., Magnusson, C., Augustsson, P., Lilja, H. & Laurell, T. Acoustofluidic, label-free separation and simultaneous concentration of rare tumor cells from white blood cells. *Anal Chem* **87**, 9322-9328, doi:10.1021/acs.analchem.5b02023 (2015).
131 Urbansky, A., Lenshof, A., Dykes, J., Laurell, T. & Scheding, S. Affinity-Bead-Mediated Enrichment of CD8+ Lymphocytes from Peripheral Blood Progenitor Cell Products Using Acoustophoresis. *Micromachines* 7, doi:10.3390/mi7060101 (2016).
132 Magnusson, C. *et al.* Clinical-Scale Cell-Surface-Marker Independent Acoustic Microfluidic Enrichment of Tumor Cells from Blood. *Anal Chem* **89**, 11954-11961, doi:10.1021/acs.analchem.7b01458 (2017).
133 Urbansky, A. *et al.* Rapid and effective enrichment of mononuclear cells from blood using acoustophoresis. *Sci Rep* **7**, 17161, doi:10.1038/s41598-017-17200-9 (2017).
134 Yasuda, K. *et al.* Using acoustic radiation force as a concentration method for erythrocytes. *The Journal of the Acoustical Society of America* **102**, 642-645, doi:10.1121/1.421009 (1997).
135 Nam, J., Lim, H., Kim, D. & Shin, S. Separation of platelets from whole blood using standing surface acoustic waves in a microchannel. *Lab Chip* **11**, 3361-3364, doi:10.1039/c1lc20346k (2011).
136 Lee, K., Shao, H., Weissleder, R. & Lee, H. Acoustic Purifiction of Extracellular Microvesicles. *ACSNano* **9**, 2321-2327 (2015).
137 Chen, Y. *et al.* High-throughput acoustic separation of platelets from whole blood. *Lab Chip* **16**, 3466-3472, doi:10.1039/c6lc00682e (2016).
138 Ngamsom, B. *et al.* On-chip acoustophoretic isolation of microflora including S. typhimurium from raw chicken, beef and blood samples. *J Microbiol Methods* **123**, 79-86, doi:10.1016/j.mimet.2016.01.016 (2016).

139 Li, S. *et al.* Acoustofluidic bacteria separation. *J Micromech Microeng* **27**, doi:10.1088/1361-6439/27/1/015031 (2017).
140 Silva, R. *et al.* Rapid prototyping and parametric optimization of plastic acoustofluidic devices for blood-bacteria separation. *Biomed Microdevices* **19**, 70, doi:10.1007/s10544-017-0210-3 (2017).
141 Wu, Y., Kanna, M. S., Liu, C., Zhou, Y. & Chan, C. K. Generation of Autologous Platelet-Rich Plasma by the Ultrasonic Standing Waves. *IEEE Trans Biomed Eng* **63**, 1642-1652, doi:10.1109/TBME.2015.2449832 (2016).
142 Wu, M. *et al.* Isolation of exosomes from whole blood by integrating acoustics and microfluidics. *Proc Natl Acad Sci U S A* **114**, 10584-10589, doi:10.1073/pnas.1709210114 (2017).
143 Dyson, M., Woodward, B. & Pond, J. B. Flow of Red Blood Cells stopped by Ultrasound. *Nature* **232**, 573-574 (1971).
144 Baker, N. V. Segregation and Sedimentation of Red Blood Cells in Ultrasonic Standing Waves. *Nature* **239**, 398-399 (1972).
145 Cousins, C. M. *et al.* Plasma preparation from whole blood using ultrasound. *Ultrasound in Med. & Biol.* **26**, 881-888 (2000).
146 Yasuda, K., Umemura, S.-i. & Takeda, K. Concentration and fractionation of small particles in Liquid by ultrasound. *Jpn. J. Appl. Phys.* **34**, 2715-2720 (1995).
147 Petersson, F., Nilsson, A., Holm, C., Jonsson, H. & Laurell, T. Continuous separation of lipid particles from erythrocytes by means of laminar flow and acoustic standing wave forces. *Lab Chip* **5**, 20-22, doi:10.1039/b405748c (2005).
148 Petersson, F., Åberg, L., Swärd-Nilsson, A. M. & Laurell, T. Free flow acoustophoresis: Microfluidic-based mode of particle and cell separation. *Anal Chem* **79**, 5117-5123 (2007).
149 Nilsson, A., Petersson, F. & Laurell, T. in *Whole blood plasmapheresis using acoustic separation chips, μTAS 2006 Tokyo, Japan.*
150 HealthCare, E. D. f. t. Q. o. M. Guide to the preparation, use and quality assurance of blood components. (France, 2015).
151 Järås, K. *et al.* Reverse-phase versus sandwich antibody microarray, technical comparsion from a clinical perspective. *Anal Chem* **79**, 5817-5825 (2007).
152 Wild, D. *The Immunoassay Handbook*. 4 th edn, (Elsevier Inc., 2005).
153 Ohlsson, P. *et al.* Integrated Acoustic Separation, Enrichment, and Microchip Polymerase Chain Reaction Detection of Bacteria from Blood for Rapid Sepsis Diagnostics. *Anal Chem* **88**, 9403-9411, doi:10.1021/acs.analchem.6b00323 (2016).
154 Ley, M. W. & Bruus, H. Continuum modeling of hydrodynamic particle-particle interactions in microfluidic high-concentration suspensions. *Lab Chip* **16**, 1178-1188, doi:10.1039/c6lc00150e (2016).
155 Li, C., Liu, C., Xu, Z. & Li, J. The dual role of deposited microbead plug (DMBP): a blood filter and a conjugate reagent carrier toward point-of-care microfluidic immunoassay. *Talanta* **97**, 376-381, doi:10.1016/j.talanta.2012.04.048 (2012).

156 Kumar, A. *et al.* Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Critical Care Medicine* **34**, 1589-1596 (2006).
157 Kellogg, J. A., Manzella, J. P. & Bankert, D. A. Frequency of Low-Level Bacteremia in Children from Birth to Fifteen Years of Age. *journal of Clinical microbiology* **38**, 2181-2185 (2000).
158 Kellogg, J. A. *et al.* Clinical Comparison of Isolator and Thiol Broth with ESP Aerobic and Anaerobic Bottles for Recovery of Pathogens from Blood. *Journal of Clinical microbiology* **32**, 2050-2055 (1994).
159 Dorn, G. L., Land, G. A. & Wilson, G. E. Improved Blood Culture Technique Based on Centrifugation: Clinical Evaluation. *Journal of Clinical microbiology* **9**, 391-396 (1979).
160 Cheng, I. F., Lin, C. C., Lin, D. Y. & Chang, H. C. A dielectrophoretic chip with a roughened metal surface for on-chip surface-enhanced Raman scattering analysis of bacteria. *Biomicrofluidics* **4**, doi:10.1063/1.3474638 (2010).
161 Park, S., Zhang, Y., Wang, T. H. & Yang, S. Continuous dielectrophoretic bacterial separation and concentration from physiological media of high conductivity. *Lab Chip* **11**, 2893-2900, doi:10.1039/c1lc20307j (2011).
162 Kuczenski, R. S., Chang, H. C. & Revzin, A. Dielectrophoretic microfluidic device for the continuous sorting of Escherichia coli from blood cells. *Biomicrofluidics* **5**, 32005-3200515, doi:10.1063/1.3608135 (2011).
163 Bisceglia, E. *et al.* A generic and label free method based on dielectrophoresis for the continuous separation of microorganism from whole blood samples. *Sensors and Actuators B: Chemical* **212**, 335-343, doi:10.1016/j.snb.2015.02.024 (2015).
164 Wu, Z., Willing, B., Bjerketorp, J., Jansson, J. K. & Hjort, K. Soft inertial microfluidics for high throughput separation of bacteria from human blood cells. *Lab Chip* **9**, 1193-1199, doi:10.1039/b817611f (2009).
165 Hou, H. W., Bhattacharyya, R. P., Hung, D. T. & Han, J. Direct detection and drug-resistance profiling of bacteremias using inertial microfluidics. *Lab Chip* **15**, 2297-2307, doi:10.1039/c5lc00311c (2015).
166 Faridi, M. A. *et al.* Elasto-inertial microfluidics for bacteria separation from whole blood for sepsis diagnostics. *J Nanobiotechnology* **15**, 3, doi:10.1186/s12951-016-0235-4 (2017).
167 Wei Hou, H. *et al.* A microfluidics approach towards high-throughput pathogen removal from blood using margination. *Biomicrofluidics* **6**, 24115-2411513, doi:10.1063/1.4710992 (2012).
168 Hou, H. W. *et al.* Broad spectrum immunomodulation using biomimetic blood cell margination for sepsis therapy. *Lab Chip* **16**, 688-699, doi:10.1039/c5lc01110h (2016).
169 Xia, N. *et al.* Combined microfluidic-micromagnetic separation of living cells in continuous flow. *Biomed Microdevices* **8**, 299-308, doi:10.1007/s10544-006-0033-0 (2006).

170 Pereiro, I. *et al.* A new microfluidic approach for the one-step capture, amplification and label-free quantification of bacteria from raw samples. *Chem Sci* **8**, 1329-1336, doi:10.1039/c6sc03880h (2017).
171 Kang, J. H. *et al.* An extracorporeal blood-cleansing device for sepsis therapy. *Nat Med* **20**, 1211-1216, doi:10.1038/nm.3640 (2014).
172 Lee, J. J. *et al.* Synthetic ligand-coated magnetic nanoparticles for microfluidic bacterial separation from blood. *Nano Lett* **14**, 1-5, doi:10.1021/nl3047305 (2014).
173 Pitt, W. G. *et al.* Rapid separation of bacteria from blood-review and outlook. *Biotechnol Prog* **32**, 823-839, doi:10.1002/btpr.2299 (2016).
174 Ai, Y., Sanders, C. K. & Marrone, B. L. Separation of Escherichia coli bacteria from peripheral blood mononuclear cells using standing surface acoustic waves. *Anal Chem* **85**, 9126-9134, doi:10.1021/ac4017715 (2013).
175 Deshmukh, S., Brzozka, Z., Laurell, T. & Augustsson, P. Acoustic radiation forces at liquid interfaces impact the performance of acoustophoresis. *Lab Chip* **14**, 3394-3400, doi:10.1039/c4lc00572d (2014).
176 Karlsen, J. T., Augustsson, P. & Bruus, H. Acoustic Force Density Acting on Inhomogeneous Fluids in Acoustic Fields. *Phys Rev Lett* **117**, 114504, doi:10.1103/PhysRevLett.117.114504 (2016).
177 Hwang, K.-Y. *et al.* Rapid detection of bacterial cell from whole blood: Integration of DNA sample preparation into single micro-PCR chip. *Sensors and Actuators B: Chemical* **154**, 46-51, doi:10.1016/j.snb.2009.11.005 (2011).
178 Cai, D., Xiao, M., Xu, P., Xu, Y. C. & Du, W. An integrated microfluidic device utilizing dielectrophoresis and multiplex array PCR for point-of-care detection of pathogens. *Lab Chip* **14**, 3917-3924, doi:10.1039/c4lc00669k (2014).
179 Kumar, A. *et al.* Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. *Chest* **136**, 1237-1248, doi:10.1378/chest.09-0087 (2009).
180 Bloos, F. *et al.* Evaluation of a polymerase chain reaction assay for pathogen detection in septic patients under routine condition: an observational study. *PLoS One* **7**, e46003, doi:10.1371/journal.pone.0046003 (2012).
181 Riegger, L. *et al.* Single-step centrifugal hematocrit determination on a 10-$ processing device. *Biomed Microdevices* **9**, 795-799, doi:10.1007/s10544-007-9091-1 (2007).
182 Thompson, B. L. *et al.* Hematocrit analysis through the use of an inexpensive centrifugal polyester-toner device with finger-to-chip blood loading capability. *Anal Chim Acta* **924**, 1-8, doi:10.1016/j.aca.2016.04.028 (2016).
183 Berry, S. B., Fernandes, S. C., Rajaratnam, A., DeChiara, N. S. & Mace, C. R. Measurement of the hematocrit using paper-based microfluidic devices. *Lab Chip* **16**, 3689-3694, doi:10.1039/c6lc00895j (2016).
184 Guhr, G. *et al.* in *Novel sensor combining impedance spectroscopy and surface acoustic waves to detect blood coagulation time and hematocrit value, Sensors IEEE 2011, Limerick, Irland.*

185 Lee, H. Y., Barber, C., Rogers, J. A. & Minerick, A. R. Electrochemical hematocrit determination in a direct current microfluidic device. *Electrophoresis* **36**, 978-985, doi:10.1002/elps.201400466 (2015).
186 Rudolf, J., Douglass, J., Baron, J. & Lewandrowski, K. Evaluation of the i-STAT point-of-care capillary whole blood hematocrit and hemoglobin: Comparison to the Siemens RAPIDLab 1200, Sysmex XE5000, and manual spun hematocrit. *Clin Chim Acta* **446**, 37-42, doi:10.1016/j.cca.2015.03.035 (2015).
187 Brimhall, O. D., Peterson, S. C., Baker, C. D. & Riddle, M. D. Apparatus and method for using ultrasound to determine hematocrit. US patent (1989).
188 Corey, S., Sakers, J., Lipford, K., Reed, S. & Murphy, B. Method and apparatus for ultrasonic determination of red blood cell indices. US patent (2010).
189 Secomski, W., Nowicki, A., Guidi, F., Tortoli, P. & Lewin, P. A. Noninvasive in vivo measurements of hematocrit. *J Ultrasound Med* **22**, 375-384 (2003).
190 Petersson, K. *et al.* Acoustofluidic hematocrit determination. *Anal Chim Acta* **1000**, 199-204, doi:10.1016/j.aca.2017.11.037 (2018).
191 Grenvall, C., Antfolk, C., Bisgaard, C. Z. & Laurell, T. Two-dimensional acoustic particle focusing enables sheathless chip Coulter counter with planar electrode configuration. *Lab Chip* **14**, 4629-4637, doi:10.1039/c4lc00982g (2014).