Label-free processing of stem cell preparations by acoustophoresis

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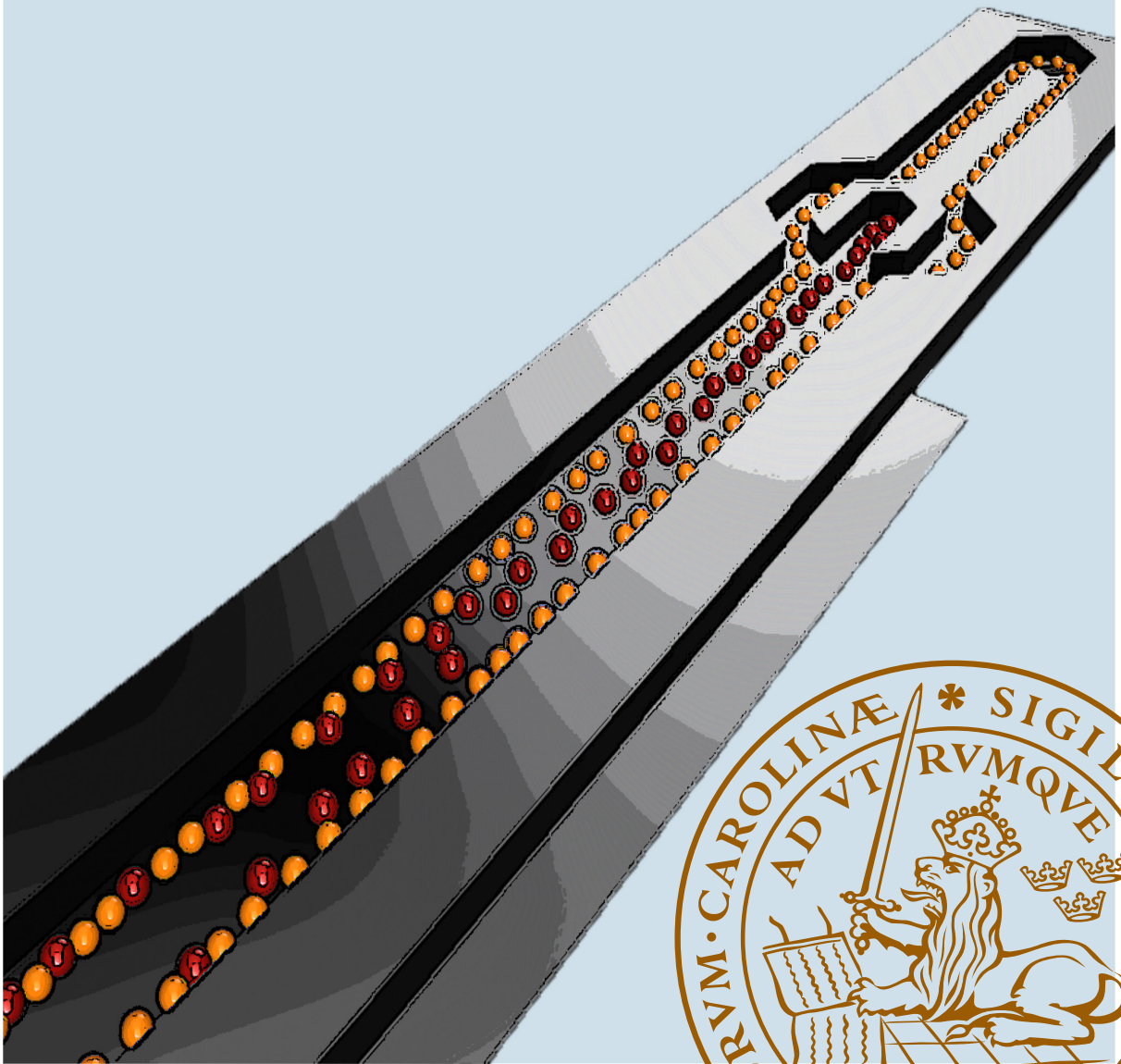

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Into a Bright Future

The regenerative capacity within the body offers great potential for the transformation into new cellular therapies. This healing power from within promises valuable treatment for many health conditions and diseases. However, challenges remain for the implementation and translation into the clinical setting. Innovations in cell processing and stem cell research can pave the way for truly translational research and clinical integration. If we stay curious and keep on imagining, we can appreciate the endless possibilities provided by the exciting times ahead.

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DOCTORAL DISSERTATION

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Abstract

The first bone marrow transplant in a human was performed in 1959, providing evidence that cells can be used for transplantation and treatment, revealing that healing capacities lie within the human body that ought to be understood. Since then hematopoietic stem cell transplantation has developed as a standard treatment for many cancers besides other malignancies. Much research is done to understand and utilize the properties of stem cells and their progenies for clinical application and transplantation. A routinely used valuable non-invasive information source in research and clinical applications is whole blood. However, cell processing including isolation or removal of certain components is desirable for many diagnostics, research, and transplantation applications.

This thesis aimed to develop and evaluate the use of a microfluidic technology, called acoustophoresis for processing human blood and bone marrow cell preparations. Acoustophoresis utilizes the phenomenon that cells can be manipulated in an ultrasonic standing wave field in microfluidic devices. In the acoustic wave field cells experience an induced movement based on their acoustophysical properties, either to the channel center (pressure node) or towards the channel walls (pressure anti-node). These properties include size, density, and compressibility also in relation to the suspending medium. This can be utilized as biophysical biomarkers in acoustic separations.

In the first two articles, it has been demonstrated that neuroblastoma tumor cells (cell line and neuroblastoma patient derived-xenograft cells) could be isolated from peripheral blood and progenitor cell products without the use of labeling antibodies, which is an advantage of acoustophoresis compared to other methods. The clinical relevance for stem cell graft processing was furthermore validated by acoustophoretic removal of transplant-contaminating tumor cells ("purging") applicable for diagnostic, prognostic as well as potentially therapeutic purposes, with preserved high cell viability and functions.

Moreover, bone marrow stromal cells (BM-MSCs) could be acoustically separated based on specific properties of primary, uncultured BM-MSCs as well as cultured MSCs. In article III, proof-of-principle evidence has been provided for the acoustic separation of functionally different subsets of cultured MSCs, which provides a first step towards better characterized and possibly enhanced cell products for cellular therapies in the future. It has further been demonstrated in article IV that primary stromal cells can be enriched from BM preparations based on their distinct biophysical properties.

For clinically relevant cell processing, a stable system with relevant throughput is required. Hence, in article V a new chip holder design with an improved air-cooling unit is presented and validated, providing improved heat distribution along with stable multiplexed separation of beads and leukocyte subpopulations. This was realized at increased sample throughput of 500 µL/min and 300 µL/min, respectively. To facilitate the development of acoustofluidic cell separation applications it is crucial to obtain the properties of cell populations of interest to predict separability together with optimal experimental conditions. Therefore, the study in article VI presents a method to statistically estimate cell compressibility based on acoustophoretic separation data.

Collectively, the work at hand provided valuable progress towards the validation and implementation of acoustic blood as well as stem cell processing in clinically relevant applications for cell transplantation, diagnostics, and regenerative medicine.

Key words: Acoustophoresis, label-free cell separation, ultrasound, microfluidics, stem cell processing, CTC separation

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To the progress created by science
and the very special people in my life.

"What you do makes a difference,
you have to decide what kind of difference you want to make."
– Jane Goodall

Table of Contents

Articles included in the Thesis

I. **Label-free neuroblastoma cell separation from hematopoietic progenitor cell products using acoustophoresis - towards cell processing of complex biological samples**
Olm F, Urbansky A, Dykes JH, Laurell T, Scheding S.
Scientific Reports, 2019, 9(1), 8777.

II. **Label-free separation of neuroblastoma patient-derived xenograft (PDX) cells from hematopoietic progenitor cell products by acoustophoresis**
Olm F, Panse L, Dykes J, Bexell D, Laurell T, Scheding S.
Manuscript

III. **Acoustophoresis enables label-free separation of functionally different subsets of cultured bone marrow stromal cells**
Olm F, Lim HC, Schallmoser K, Strunk D, Laurell T, Scheding S.
Submitted manuscript (2020)

IV. **Acoustophoretic label-free enrichment of primary stromal cells from bone marrow samples**
Olm F, Löffler P, Vlieghe H, Laurell T, Scheding S.
Manuscript

V. **Label-free separation of leukocyte subpopulations using high throughput multiplex acoustophoresis**
Urbansky A, Olm F, Scheding S, Laurell T, Lenshof A.
Lab on a Chip. 2019, 19(8), 1406-16.

VI. **Statistic Estimation of Cell Compressibility Based on Acoustophoretic Separation Data**
Garofalo F, Lenshof A, Urbansky A, Olm F, Bonestroo A, Ekblad L, Scheding S, Laurell L.
Submitted manuscript (2020)

Preface

We do live in exciting times with advances in biomedicine being made every day. New technologies are developed and harnessed to perform research enabling us to understand more about how cells work and interact, how we can utilize the knowledge to develop new treatments, fight diseases as well as how to benefit from the tools and information that lay within us. The human body has remarkable properties including self-healing capacities. On a daily basis, over hundred billion cells are replaced through the blood building system, liters of fluids are pumped through each corner of the body to maintain all life functions, without us even consciously thinking about it. If tissue is damaged it can often heal by itself. Even after severe damage, *e.g.* after radiation or chemotherapy, the blood building system can be restored. All thanks to the existence and transplantation of some rare cells found throughout our bodies in places like the bone marrow.

Yet, there are still opportunities for improvement in many connected fields, including the methods for separating cells of interest from complex mixtures. Necessary separation outcomes, such as purity and recovery, might vary depending on the application but common is the need to preserve cell viability and functions.

In recent years the field of microfluidic cell manipulation has expanded extensively while creating many solutions for various applications such as separation, sorting, concentration, washing, alignment, as well as trapping of cells or particles which can be used stand-alone or combined in unit operations.

I hope this thesis provides some new useful insights; a knowledge piece in the research puzzle towards the translation of acoustophoresis, as a microfluidic technology, to its routine use for blood and stem cell processing in research as well as clinical applications.

If you are looking for a “short and sweet” summary of this thesis, you can find a popular science summary on page 69 pp.

Abstract

The first bone marrow transplant in a human was performed in 1959, providing evidence that cells can be used for transplantation and treatment, revealing that healing capacities lie within the human body that ought to be understood. Since then hematopoietic stem cell transplantation has developed as a standard treatment for many cancers besides other malignancies. Much research is done to understand and utilize the properties of stem cells and their progenies for clinical application and transplantation. A routinely used valuable non-invasive information source in research and clinical applications is whole blood. However, cell processing including isolation or removal of certain components is desirable for many diagnostics, research, and transplantation applications.

This thesis aimed to develop and evaluate the use of a microfluidic technology, called acoustophoresis for processing human blood and bone marrow cell preparations. Acoustophoresis utilizes the phenomenon that cells can be manipulated in an ultrasonic standing wave field in microfluidic devices. In the acoustic wave field cells experience an induced movement based on their acoustophysical properties, either to the channel center (pressure node) or towards the channel walls (pressure anti-node). These properties include size, density, and compressibility also in relation to the suspending medium. This can be utilized as biophysical biomarkers in acoustic separations.

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stromal cells can be enriched from BM preparations based on their distinct biophysical properties.

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List of selected Abbreviations

7-AAD	7-amino-actinomycin D
ACDA	Anticoagulant Citrate Dextrose Solution A
ANOVA	Analysis of variance
BAW	Bulk acoustic wave
BioMEMS	Biomedical microelectromechanical systems
BM	Bone marrow
BM-MNC	Bone marrow mononuclear cell
CFU-F	Colony forming unit-fibroblast
CTC	Circulating tumor cell
DEP	Dielectrophoresis
DLD	Deterministic lateral displacement
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
$\mathbf{E_{ac}}$	Acoustic energy density
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
$\mathbf{F_{rad}}$	Primary acoustic radiation force
G-CSF	Granulocyte-colony stimulating factor
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
LC	Lymphocyte

MACS	Magnetic-activated cell sorting
MNC	Mononuclear cell
MRD	Minimal residual disease
MSC	Mesenchymal stromal (stem) cell
NB	Neuroblastoma
NBC	Neuroblastoma cell
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PBPC	Peripheral blood progenitor cell
PBPCP	Peripheral blood progenitor cell product
PBS	Phosphate buffered saline
PDX	Patient-derived xenograft
pHPL	Pooled human platelet lysate
PI	Propidium iodide
PRP	Primary radiation force
PS	Polystyrene
PZT	Piezoceramic transducer
RBC	Red blood cell
Re	Reynolds number
SAW	Surface acoustic wave
SC	Stem cell
SD	Standard deviation
SSR	Side stream recovery
WBC	White blood cell

Introduction

Cell-based therapies came a long way from the first human bone marrow transplant in 1959. E. Donnall Thomas provided the first evidence that bone marrow cells could reconstitute the blood building system after lethal irradiation of patients with acute leukemia[1]. Nowadays, hematopoietic stem cell transplantation (HSCT) is a standard treatment while advanced gene editing and personalized cellular therapies are emerging. Cells for stem cell transplantation and research are commonly obtained from bone marrow, peripheral blood, or cord blood. They can be utilized from the patient (autologous), from family donors, or unrelated voluntary donors (allogeneic).

Apart from using stem cells for HSCTs, research and clinical development are performed to understand the role of mesenchymal stromal and other (stem) cells from tissues in their residing location in the body, their role in diseases as well as their use in regenerative medicine.

Moreover, the use of blood and its components is routinely implemented in the clinic for diagnostic, analytical, and therapeutic purposes. Further development is an ongoing process. All these examples, however, have in common that to utilize and further understand their applications, functions, especially the opportunities lying in their different components, the ability to isolate cells from complex mixtures in biofluids is required.

This has traditionally been realized using macroscale separation methods, but novel microscale fluidic methods are emerging in life sciences and cell processing. These methods offer new opportunities in precise, efficient, and gentle cell separation, as well as isolation. The research included in this thesis investigates the use of acoustophoresis, a microfluidic technology that uses acoustic forces in the form of ultrasonic standing wave fields in microchannels, for the processing of stem cell and blood preparations. Considerations towards the application of the technology in research and clinical settings in different relevant contexts are described.

This chapter intends to summarize background knowledge and outlay the foundation for the work included in this thesis. Relevant blood and stem cell types, their functions, origin, and applications will be briefly covered as well as selected macroscale and microscale cell separation methods along with their features. Moreover, acoustophoresis together with its underlying basic principles are described in more detail.

Hematopoiesis

Hematopoiesis describes the continuous formation and development of blood cells. It is a dynamic, complex process whereby a very rare population of hematopoietic stem cells (HSCs) residing in the bone marrow give rise to committed progenitor cells (myeloid or lymphoid precursors), which differentiate into more mature cells, ultimately building and restoring all blood cells in the body (Figure 1). This is possible due to HSCs multipotency as well as their capacity to self-renew. HSCs reside in the body throughout life and have the ability for asymmetric cell division. Allowing them to give rise to highly proliferating, gradually differentiating progenitor cells, ensuring continuous replenishment of mature blood cells. During maturation, the self-renewal capacity of the progeny is decreasing progressively and lineage differentiation becomes more restricted.[2]

Hematopoietic stem cells and peripheral blood

HSCs are the origin of all blood cells. and to date one of the best-characterized stem cells in the body. They are the only stem cell type that is routinely applied in the clinic for the treatment of various malignancies and therapies, including blood cell disorders, in cancer therapy to restore the hematopoietic system, as well as congenital immunodeficiencies.[2] HSCs, residing in a tightly regulated niche in the bone marrow, give rise to multipotent myeloid and lymphoid progenitor cells that further differentiate into committed precursors and finally into highly specialized mature blood cell types with important roles in the immune system and tissue repair. Lymphoid progenitors give rise to T, B, as well as NK cells, whereas myeloid progenitors eventually further differentiate into granulocytes, monocytes, macrophages, erythrocytes, dendritic cells, and megakaryocytes (Figure 1).

Blood is a remarkable, specialized body fluid that consists of plasma, red blood cells, white blood cells, and platelets as main components. It accounts for approximately 7-8% of the body weight, whereby, about 55% are blood plasma, the other 45% are cells. Blood has many different functions, such as oxygen and nutrient transportation to the lungs and tissues, as well as transportation of waste products to the kidneys and liver for filtration and cleaning. Blood carries cells and antibodies that fight infections, clots are formed for wound healing, to prevent blood loss. Furthermore, it regulates the body temperature. Blood *plasma* is the liquid component, a mixture of water, electrolytes, glucose, proteins, lipids as well as other soluble substances. The plasma enables transport of cells, nutrients, waste products, antibodies, various proteins, as well as chemical messengers and maintains the body's fluid balance to sustain physiological processes.

Red blood cells or erythrocytes (RBCs), transport oxygen from the lungs to the tissues, where it is exchanged for carbon dioxide and returned to the lungs. RBCs are the most abundant cell type in whole blood with about 90%. RBCs originate from nucleated cells but shed their nuclei as they transition from the marrow sinuses to peripheral blood, thus obtaining the characteristic biconcave disc shape. This provides the advantage of reduced weight, a larger volume to surface area ratio for increased gas exchange, as well as increased deformability.

White blood cells or leukocytes (WBCs) account for ~0.1% of cells in the blood, play important roles in the immune system, and defend the body against infections, microorganisms, as well as other foreign offenses.

Lymphocytes are essential for immunity through their ability to recognize, bind pathogen antigens, and trigger the immune response. They account for ~30% of leukocytes in the blood and can be further divided into more specialized cells. *T-lymphocytes* (~75% of lymphocytes) are the basis of cell-mediated immunity. Their T-cell receptors require antigen-presenting cells that express human leukocyte antigens (HLA). T-cells are further divided into *T helper cells*, which release cytokines ($CD4^+$), and *cytotoxic T-cells* ($CD8^+$) that produce toxic substances to induce death of infected cells. *B cells* are part of the humoral immunity of the adaptive immune system, secrete antibodies along with cytokines, and present antigens. *Natural killer cells* (NK cells) are part of the innate immune system and act quickly against several pathological challenges. NK cells kill virally infected cells, detect as well as control early signs of cancer, and protect the body against other diseases.

Phagocytes, such as granulocytes and monocytes engulf and destroy damaged cells as well as foreign material as part of the innate immune response. *Granulocytes* account for ~65% of WBCs and are morphologically characterized by inclusions or granules in their cytoplasm which contain enzymes or toxins to kill target cells. Granulocytes can be further divided into neutrophils, eosinophils, and basophils.

Neutrophils (~95.5% of granulocytes) are the most abundant leukocyte cell type and one of the first cell responses in the defense against invading microbes. They act by phagocytosis or release of antimicrobial factors from their granules. Moreover, they secrete a set of cytokines and chemokines that have pro-inflammatory as well as immunomodulatory effects, recruit other immune cells through chemotaxis, and further interact with other immune and non-immune cell types. *Eosinophils* play important roles in the pathogenesis of various allergenic, neoplastic, and parasitic disorders. They have proinflammatory as well as cytotoxic activity. *Basophils* account for less than 1% of circulating leukocytes. They contain cytoplasmic granules containing mediators of acute inflammation, such as heparin and histamine.

Monocytes, accounting for ~5% of leukocytes, circulate in the blood from where they migrate to tissues and further differentiate into macrophages as well as dendritic cells. Apart from playing important roles in the nonspecific immune response, *macrophages* present antigens to T-lymphocytes, secrete numerous cytokines involved in immunity,

inflammation as well as hematopoiesis. *Dendritic cells* are also antigen-presenting cells, linking the innate with the adaptive immune response by T-cell activation.

Platelets or thrombocytes are small disc-shaped cell fragments produced in the cytoplasm of megakaryocytes. Platelets are involved in blood clotting, which is a critical defense mechanism of the body and protects the vascular system after injury in conjunction with inflammatory and repair responses. Bleeding is stopped at the site of damaged endothelium by platelet adhesion and activation, whereby platelets change to a spherical shape, secrete chemical messengers, and connect through receptors for aggregation, leading to the formation of a platelet plug and the initiation of a coagulation cascade.[3,4]

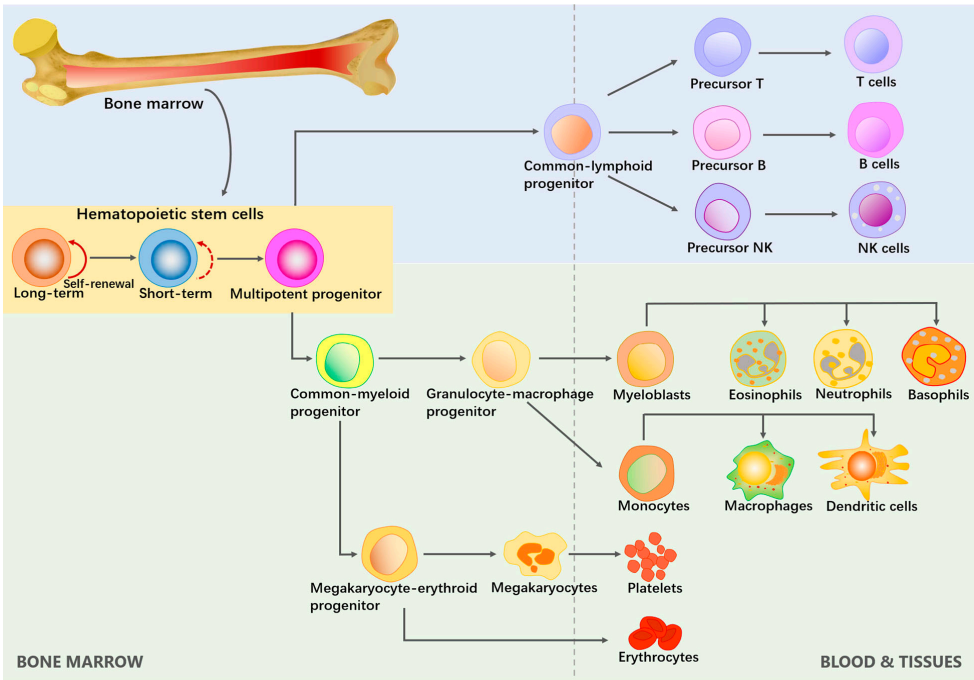

Figure 1: Schematic overview of hematopoiesis. Presented are HSC self-renewal and development to mature blood cells through a series of gradually more lineage-restricted progenies. Reprinted with permission from Zhang *et al.*[5], © 2019, Springer Nature.

Blood as an important diagnostic and therapeutic source

Blood is extensively used as diagnostic as well as therapeutic source in the clinic. Massive amounts of information from every tissue or organ are contained in blood, due to its continuous circulation through each corner of the body, combined with its role as carrier for nutrients and messengers. Thus, blood analysis provides important indications for the

diagnosis of many physiologic and pathologic conditions. The contained cells are indicators of disease status as well as immune response; some hold great therapeutic potential. Hence, blood plays a central role in the development of clinical and research applications.

Complete blood counts (CBC) are routinely used tests to evaluate the overall health or disease status of a person. CBCs include measurements of blood properties and components, such as RBCs as well as their ratio to the plasma (hematocrit), hemoglobin, WBC, and platelet counts. A change in blood features or cell counts are common accompaniments of various disease states and disorders, such as infection, anemia, or leukemia. Cell-free plasma is used for biomarker analysis, *e.g.* in early cancer detection. Blood cultures are commonly used to detect bacterial or fungal infections. Furthermore, circulating tumor cells (CTCs) can be isolated from blood to serve as important diagnostic, prognostic, as well as disease monitoring tools. The isolated cells can be analyzed molecularly, used for drug testing or for the development of personalized therapies[6,7]. Moreover, rare fetal cells can be isolated from maternal blood serving as non-invasive prenatal diagnostics[8].

Whole blood, as well as its components, are used as therapeutics and considerable research is done to develop cell-based therapies. For example, different leukocyte populations are isolated, subsequentially differentiated or modified, and eventually retransfused to the patient. WBCs are also employed for DNA sequencing as well as several hematological tests. Furthermore, platelet transfusion is routinely used for treating or preventing complications associated to bleeding, *e.g.* in connection to surgery, or disorders involving abnormal platelet counts. Granulocyte transfusions are applied in neutropenic patients with severe bacterial or fungal infections.[3,9]

Hematopoietic stem cell transplantation

Historically, bone marrow cells were used to reconstitute the blood building system after irradiation, which was the groundwork for cell therapies. This breakthrough in modern medicine was later recognized with a Nobel Prize in 1990[10,11]. Today, HSCT is a well-established and successfully utilized therapy to treat many life-threatening cancers, including selected solid tumors, malignant, as well as non-malignant disorders. Stem cells can be obtained from bone marrow (BM), peripheral blood, or cord blood from either the patient, family members, or unrelated donors. Currently, in the majority of HSCT procedures, peripheral blood progenitor cells are the stem cell source of choice because of their availability, non-invasiveness of the collection procedure, along with the favorable engraftment compared to BM. PBPCs are mobilized from the bone marrow to the peripheral blood by administration of hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF). This results in sufficient numbers of PBPCs in the circulation for collection by standard apheresis procedures.[12,13]

Autologous HSCT is employed after hematotoxic high-dose radio or chemotherapy to restore hematopoietic function without the concern of graft-versus-host disease (GvHD).

Allogeneic HSCT, using PBPCs from an HLA-matched donor, is mainly used in high-risk or relapsed acute leukemia. Herein, the transplanted cells do not only have the potential to restore the hematopoietic system but also to renew the immune system. This can mediate transplant-versus-malignancy effects, however with a risk for potentially lethal GvHD.

PBPCs are identified and enumerated by their $CD34^+$ surface marker expression, which is detectable on the majority of cells with hematopoietic reconstitution potential. The minimum dose of $CD34^+$ cells for HSCT is $2\text{-}5\times10^6$/kg bodyweight of the recipient. Hence, several apheresis collections may be required to achieve sufficient cell numbers for transplantation, often along with the need to freeze cell collections for storage. Cord blood cells that were collected and stored at birth, can be an alternative allogeneic stem cell source for HSCT.[14-16]

Apheresis cell collection procedure

For HSCTs, PBPCs are routinely collected by apheresis procedures following the patient's mobilization treatment with G-CSF[12,17,18]. Most commonly, the apheresis technology is based on continuous flow centrifugation of anticoagulated peripheral blood. Mononuclear cells are collected while the other remaining blood components are returned to the patient or donor.[13,19,20] The collection efficiency of PBPCs is about 50%, which often induces the need for extended apheresis procedures (~4-5 hours). Cell collection might be necessary over several consecutive days to collect the required dose of $CD34^+$ cells for transplantation. Even after reaching sufficient PBPC numbers the cell product only consists of 1-5% PBPCs along with various other leukocyte populations.[20-26]

Neuroblastoma

Neuroblastoma (NB) is an early childhood cancer that is the most common malignancy in children diagnosed within the first year of life. It is one of the most aggressive extracranial solid tumors that arises in the sympathetic nervous system during development. The condition accounts for up to 15% of death in pediatric cancers with a mean average age of 18 months at diagnosis. NB characteristics are extremely heterogeneous ranging from spontaneous regression in low-risk patients to malignant progression along with relapse in high-risk patients. The heterogeneity is also presented in the tumor's anatomical localization, histology, clinical manifestations as well as the genomic and molecular profile.

Diagnosis is based on a combination of laboratory tests, pathology, and radiographic imaging, followed by disease staging[27]. In very young infants, spontaneous regression can often be seen in case of favorable tumor biology, even without treatment in metastatic disease.

However, for children with diagnosis after 18 months of age, with unresectable or metastatic, biologically unfavorable tumors, intensive treatment is required, with often poor prognosis along with much lower overall survival. Over 50% of newly diagnosed patients are classified as high-risk neuroblastoma with highly metastatic tumors. About

20% remain with minimal residual disease (MRD) during chemotherapy. A large part of high-risk patients will remain with MRD that causes relapse, even after additional consolidation therapy.[28,29] However, the clinical significance of MRD in neuroblastoma remains to be established. Even though great improvement has been achieved in the therapy of neuroblastoma patients, long-term survival rates remain poor at less than 50% for high-risk patients[27]. Long-term survival for relapsed patients is even worse, with less than 10%[30].[31,32]

Treatment and graft processing

Treatment for neuroblastoma is based on risk stratification after diagnosis. For low-risk neuroblastoma, treatment aims at minimum therapy to avoid treatment-related side effects for the patients. Treatment for intermediate-risk neuroblastoma often involves several cycles of chemotherapy as well as surgical resection of the tumor if possible.

For high-risk neuroblastoma patients, treatment usually involves intense multidisciplinary approaches as illustrated in Figure 2. At first, induction chemotherapy is incorporated to shrink the primary tumor and reduce metastases to minimize the overall tumor burden. HSCs are mobilized, harvested, and usually stored at -157°C. The combination chemotherapy regimen is followed by surgical resection of the primary tumor. Subsequent consolidation therapy consists of myeloablative chemotherapy supported by autologous HSCT as well as local control of the primary tumor by radiotherapy. Maintenance therapy for MRD combines immunotherapy (anti-GD2 monoclonal antibody along with cytokines) with isotretinoin[33].[15,16]

MRD refers to drug-resistant remaining tumor cells after the extensive treatment regimen. Although the clinical significance in neuroblastoma is not fully established yet, accurate, sensitive detection of MRD is a valuable tool to achieve optimal treatment outcome in high-risk patients.[27,32]

Primary cancer cells can often obtain the ability to shed from the main tumor, migrate, disseminate, invade, eventually proliferate in distant locations providing prerequisites for metastases or relapse[34]. Circulating tumor cells are detectable in peripheral blood and PBPC samples of neuroblastoma patients, as well as disseminated tumor cells in BM[35-38]. These cells are indicators for MRD dynamics and further contain valuable information of the primary tumor. As invasive tumor biopsy of primary or metastatic tumors is not always possible, less invasive sampling of peripheral blood as well as BM has established clinical relevance in many cancer types[39].

Diagnostic and prognostic CTC collection from blood or PBPCs is desirable and holds value for disease status determination as well as treatment evaluation. Sufficient tumor cell detection, recovery as well as purity are required dependent on the downstream analysis. Circulating tumor cell DNA, providing diagnostic as well as prognostic information, can also be detected and isolated from blood serum or plasma.

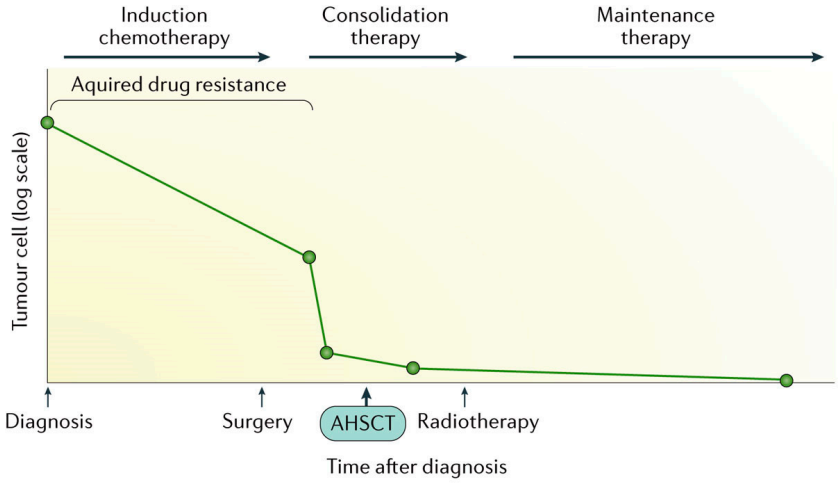

Figure 2: Overview of the treatment approach for high-risk neuroblastoma. Reprinted with permission from Matthay *et al.*[31], © Springer Nature 2016.

PBPC purging, referring to the removal of contaminating tumor cells from the graft, remains a controversial topic in pediatric oncology. As one proliferating tumor cell can be sufficient to form a new tumor, it seems apparent, that removal of graft-contaminating tumor cells is desirable to reduce relapse risks. Even though the benefit of purging for clinical outcomes is not conclusively validated.[35,40,41]

Rill *et al.*[42] demonstrated in 1994 that neomycin-resistance gene-marked tumorigenic cells were detectable in patients with relapse following BM transplantation, in as well as outside the BM. In the following years, great research efforts were allocated to establish strategies for tumor cell removal from the stem cell graft before autologous HSCT[40,43-46].

Common purging strategies include *ex-vivo* incubation with cytotoxic drugs, positive selection of $CD34^+$ cells with magnetic activated cell sorting (MACS), or affinity-based neuroblastoma cell depletion with specific antibodies. These methods are clinically applicable but tend to be time-consuming, rather expensive, usually require trained personnel, sample handling, besides processing can be disadvantageous. Furthermore, purging efficiency can be insufficient, damage or decrease of desired cells can occur. Loss of immune cell populations may increase the infection probability post transplantation[40].

Generally, more research on the impact of graft purging will be necessary, including the comparison of different purging methods and their efficiency. Nevertheless, it appears preferable to remove tumor cells from the PBPC transplant to avoid reinfusion of viable tumor cells possibly contributing to relapse of the patient. Depleted tumor cells could serve for immunotherapy approaches after irradiation or further molecular characterization of the primary tumor.[41,47,48]

Bone marrow and mesenchymal stromal cells

Bone marrow accounts for approximately 5% of the body weight in adults and is the major hematopoietic organ. It consists of red (location of hematopoiesis) and yellow (high fat content) marrow. BM tissue lies in central bone cavities, providing an ideal microenvironment for hematopoietic stem cell maintenance, growth, and development. BM contains developing blood cells as well as supporting stromal tissue.

*Mesenchymal stromal cells (*MSCs*)* represent a rare non-hematopoietic multipotent adult cell type in the bone marrow. The presence of those large, spindle-shaped, fibroblast-like cells in BM was discovered by Friedenstein and colleagues in 1966. They were later able to isolate colony-forming cells by plastic adherence, further demonstrating their multilineage differentiation potential.[49-51] The term mesenchymal stem cell was coined by Caplan in 1991[52]. Tremendous research efforts were conducted in the last decades to characterize MSCs, to understand their functions as well as their biological roles, to possibly use these cells in cell therapies.

These skeletal stem cells differentiate into adipocytes, osteocytes, as well as chondrocytes *in vitro* and *in vivo*. MSCs can serve as a feeder layer in co-cultures with HSCs to investigate the interplay between these two stem cell types, especially their hematopoietic support functions[53]. Stromal cells are a heterogeneous cell population that plays important roles during development, tissue injury, regeneration, as well as immune responses, contributing to various biological processes in different tissues. The term MSC has been used to describe stromal cells found in various tissues, such as BM, adipose tissue, dental pulp, umbilical cord blood, dermis, or lung tissue.

MSCs' multidirectional role in various tissues and organs attracted much research attention. Their key roles in fundamental biology, as well as their clinical importance and potential, become progressively apparent. Historically, MSC definitions relied on MSC properties of *in vitro* cultured BM cell preparations. As the number of publications in the MSC field increased and was further widened to cells from various sources, the International Society for Cellular Therapy (ISCT) published a position paper proposing minimal criteria for the unified definition of *in vitro* expanded mesenchymal stromal cells in 2006[54]. The main criteria, being used until today, are adherence to plastic surfaces under standard culture conditions, the surface marker expression for CD105, CD73, and CD90 as well as the lack of expression of surface molecules for CD45, CD34, CD14 or CD11b, CD19 or CD79α, and HLA-DR. Additionally, MSCs must have the ability to differentiate into adipocytes, osteoblasts, as well as chondrocytes *in vitro*. These minimal criteria provided a basis for the standardization of cultured stromal cell preparations. However, the guidelines are not sufficient to identify cells that necessarily comply with native BM-MSC properties, resulting in heterogenous cell populations *in vitro*.[55]

Mesenchymal stromal cell functions

Multipotency and self-renewal

A main characteristic of MSCs is their differentiation capacity under specific inducing culture conditions into three major mesenchymal lineages through osteogenesis, adipogenesis, and chondrogenesis[56]. The ability of MSCs to proliferate and differentiate into skeletal cells including bone as well as cartilage precursors show their potential for tissue regeneration and repair. Moreover, it has been suggested that MSCs can stimulate repair mechanisms and endogenous stem cells. MSCs may further have the capability to differentiate into other mesodermal and possibly non-mesodermal cell types, which was partly demonstrated *in vitro*, but remains controversial *in vivo*[55,57] (Figure 3).

A major criterion for MSCs is their self-renewal capacity. Only if this stemness property is proven they should be considered stem cells[58]. The clonogenic potential of stem and progenitor cells is confirmed by colony forming unit-fibroblast (CFU-F) assays or nonadherent mesensphere assays. Self-renewal is commonly demonstrated by serial transplantation of culture expanded clonal CFU-F or mesensphere into immunodeficient recipients[55,59].

Hematopoietic support

MSCs play an important role in the bone marrow. They constitute an essential HSC niche component by actively maintaining hematopoietic stem and progenitor cells as well as by supporting their functions[60]. MSCs respond dynamically through synthesis of cytokines along with growth factors to support HSC requirements.[61]

Hence, expression of genes for related factors can be an indication of MSC hematopoietic support functions. These supporting factors include the chemokine CXCL12 regulating HSC migration[62], SCF (stem cell factor) controlling HSC adhesion and quiescence[63], besides Notch ligands, contributing to HSC maintenance and self-renewal. Moreover, vascular cell adhesion molecule-1 (VCAM-1) which mediates HSC adherence, Angiopoietin-1, Osteopontin, and Thrombopoietin-1 contributing to maintained HSC quiescence, among others.[55,64]

Immunomodulation

One of the most interesting features of MSCs is their immunomodulatory functions, which have been motivating research towards their use in clinical cell-therapy studies. The ability to inhibit T-cell proliferation *in vitro* as well as their immunosuppressive actions as shown in *e.g.* skin allograft rejection models were demonstrated[65,66].

It has further been demonstrated that MSCs differentiate T-cells towards T regulatory cells (Tregs), which results in decreased inflammatory responses. These effects are being further investigated, achieving some promising results in different disease contexts, although the exact mechanisms and stromal cell population exerting these properties are still mostly unknown.

MSCs showed antiproliferative effects on B cells and affected other immune cells, such as dendritic cells, NK cells, and macrophages. They have further been shown to release anti-inflammatory substances. Another important feature of MSCs is their relatively low expression of major histocompatibility (MHC) class I antigens, which is why they are regarded as immune-tolerant cells. Hence, there is only minimal risk of rejection of MSCs following transplantation.[55]

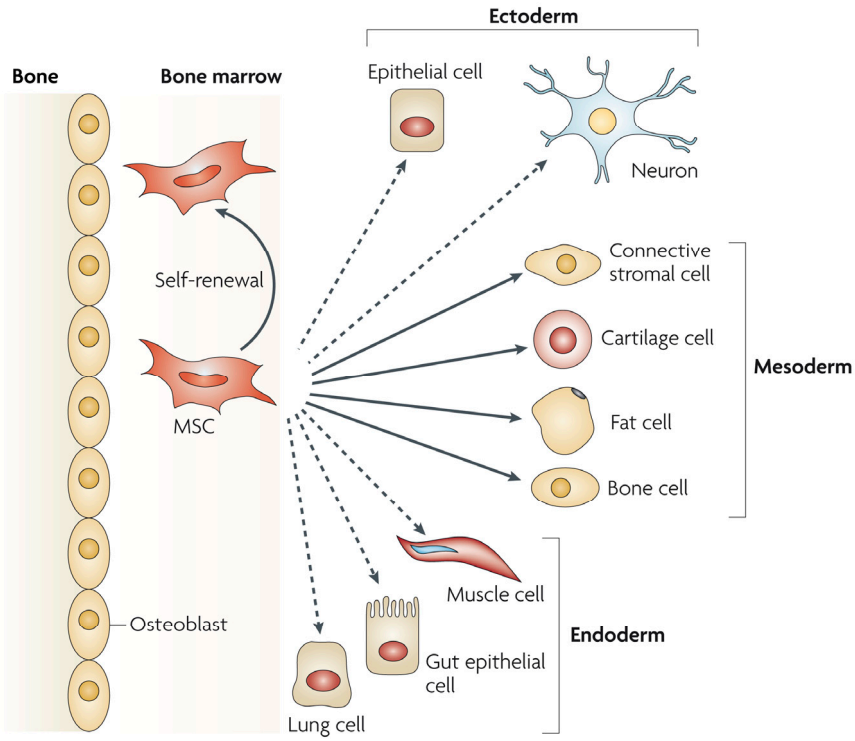

Figure 3: Schematic overview of MSC self-renewal in the bone marrow along with their differentiation capacity. Solid arrows indicate differentiation capacity of MSC towards mesodermal cell types. MSC transdifferentiation capacity towards other lineages is still controversial *in vivo* and is thus is shown in dashed arrows. Reprinted by permission from Macmillan Publishers Limited, Nature Springer, Nature Reviews. Immunology, Uccelli, A., Moretta, L. & Pistoia, V. Mesenchymal stem cells in health and disease. Nature reviews. Immunology 8, 726-736 (2008).[57], © 2008.

Clinical applications of MSCs

Life expectancies are increasing thanks to advances in medical care and treatments. This however brings new challenges, health issues, along with healthcare burdens connected to diseases primarily found in the aging population[67]. These include, aging tissue, bone trauma

or disease, as well as other degenerative disorders, thus accelerating the need for innovative regenerative and cell therapies, in addition to stem cell research.

MSCs can easily be isolated from BM or other tissues and expanded *in vitro*, while key properties are maintained for extended times in culture. The cells are suitable for autologous and allogeneic transplantation. Therefore, they are interesting candidates for cell therapies. These features have led to the rapid investigation and development of potentially interesting clinical applications exploring their differentiation as well as tissue preservation potential, anti-inflammatory abilities, and regenerative capacity.

However, there is still much to investigate and understand in MSC development, identities, functions as well as the specific contributions of cell subpopulations to these various beneficial effects. Eventually enabling to implement and harvest their full potential for cell therapies. MSC therapy has great therapeutic implications but the cell product and its detailed action mechanisms remain widely uncharacterized. Thus, hindering the approval of MSC therapies, even though numerous promising applications are under investigation in pre-clinical and clinical trials.

MSCs in tissue protection and regenerative medicine

MSCs play important roles in tissue development, maintenance, and repair. They showed great potential in musculoskeletal regenerative therapies connected to age-related degenerative diseases, and other clinical conditions. MSCs produce and secrete important factors that support engraftment in addition to trophic functions in tissue repair. *In vivo* studies with MSCs have long been focused on their HSC support functions to facilitate engraftment in HSC transplantation[68,69], along with their structural or functional tissue repair potential[70-72].

Promising results were achieved for MSC transplantations in bone diseases and repair, such as osteoarthritis, *osteogenesis imperfecta*, or in cartilage regeneration[73.74] MSC transplantation has also been investigated for the treatment of auto-immune diseases, multiple sclerosis, in addition to other neurological diseases where protective properties on injured neurons and anti-inflammatory effects were demonstrated[75-77]. Moreover, *in vivo* administration of MSCs in diabetes mouse models showed promising modulation effects on pathogenic B and T-cell response as well as the promotion of endogenous repair through bystander effects on target tissues[78,79].

Positive effects of MSCs through cytokine release were also observed in models of acute renal failure[80,81], lung fibrosis[82], and ischemic cardiomyocytes[83].[57] Furthermore, MSC treatment effects have been investigated in Crohn´s disease[84], systemic lupus, *diabetes mellitus*, *retinitis pigmentosa*, and spinal cord injury among others[85,86].

MSCs in HSCT and Graft-versus-Host disease

As aforementioned, allogeneic HSCT can lead to a severe life-threatening complication, called graft-versus-host disease, which occurs when donor allogeneic T-cells attack the recipient's organs or tissues. Co-transplantation of MSCs along with human HSCs

showed promising engraftment support, increased hematopoiesis effects in sheep studies[68], as well as accelerated BM recovery in cancer patients after HSCT[87,88]. MSCs further demonstrated promising results in several studies to support HSC engraftment while decreasing the risk of GvHD[89-93].[61]

MSCs in cancer

It is known that MSCs affect the tumor microenvironment in various ways, *e.g.* through vascular support, recruitment of immune cells, by providing a fibroblast network as well as possibly by secretion of soluble factors. However, if MSCs influence metastasis and cancer development positively or negatively is still not fully understood. The discrepancies between different studies might be due to different MSC sources or different conditions for cell expansion *in vitro*. This emphasizes the need in the field for performing studies with more homogenous MSC populations that were obtained under clinically relevant standardized conditions[94]. Other studies showed that MSCs are recruited to tumor sites, hence fuelling the idea that the cells could be employed as vehicles for drug delivery to the tumor[95,96].[97]

Biophysical properties of cells

For the development of label-free cell separation techniques, the ability to measure biophysical properties of cell populations is of great value, as it allows to predict separability of different cell types. These biophysical markers have been widely explored in cell separation technologies, but cell properties are often scarcely available in the literature, leading to trial and error approaches.

One of the most widely available biophysical parameters for commonly used cell types is cell size, as the measurement can be performed using widely accessible equipment. These methods can include filters[98], image-based measurements, as well as impedance based-flow cytometry (coulter principle[99]) or relative measurements using light scatter properties in flow cytometry.

Macroscale and microscale methods have been developed for population-based or single-cell measurements of other biophysical properties, such as density, compressibility, deformability, or electrical impedance.[100-106] Selected biophysical properties of human cell populations relevant for or obtained in the work of this thesis are summarized in Table 1.

Table 1: Overview of human cell properties[98]. *Values from Cushing *et al.*[100], **values from article V, ***values from article VI.

Cell type	Diameter [µm]	Density [g/cm^3]	Compressibility [TPa^{-1}]
WBCs	5.5-12**	1.055–1.085	402***
Lymphocytes	5.5-12**	1.055–1.070	395***
Monocytes	7.5-12**	1.055–1.070	400***
Granulocytes	8.5-11**	1.075–1.085	412***
HSCs (CD34$^+$)	7-10***	1030[107]	385***
MSCs	10-28***	1054***	385***
NBCs (SH-SY5Y)	10-22***	1059*	430***
RBCs	6-9	1.089–1.100	334*
Platelets	2-4	1.04–1.06	N.A.

Cell separation methods

Cell separation technologies are an essential part of procedures in biomedical and clinical laboratories. They play a fundamental role in the isolation, collection, as well as processing of cells and cell products, *e.g.* for hematopoietic or stem cell preparations, diagnostics, prognostics, therapeutic administration, and research purposes. Samples of interest are often complex biofluids and heterogeneous cell populations or mixtures.

Whole blood, for example, is easily accessible, rich in information but has a very complex composition of various cell types with different functions and informative values. Isolation of certain cell types or fractionation of populations is often necessary for further analysis, whereby the preservation of viability and cell function is essential. Various cell manipulation or sorting techniques have been developed at the macro- and microscale. Commonly, these separation methods can be based on biochemical labels or intrinsic biomarkers as well as a combination or sequence thereof.

For instance, to achieve primary extraction of leukocytes from bone marrow or whole blood, usually, a first separation is performed based on biophysical properties such as size and density. This is often followed by a more specific label-based method to isolate or remove selected cell types from the cell preparation. Besides, label-free methods have been developed to separate cell populations from leukocytes or other cell mixtures without the need for affinity-binding. In this way, the graft or sample composition can be altered, or selected populations of interest can be isolated for further downstream processing or analysis.

The use of cell separation procedures for clinical cell-based therapies or treatments has the potential to further improve clinical outcomes by selecting and standardizing the composition. Thus, the quality of the cell product and its efficiency could be increasing. The following section will provide an overview of selected conventional and microfluidic cell processing technologies relevant for research and clinical applications.

Conventional separation methods

Conventional cell separation methods are widely applied in biomedical and clinical laboratories for cell processing, isolation, and separation. These methods vary in labor intensity, costs, and complexity, but also in their throughput, sensitivity, as well as efficiency. Commonly utilized methods include centrifugation, flow cytometry, and affinity-based separation. Table 2 categorizes label-free and label-based methods that are summarized below.

Table 2: Overview and comparison of label-free and label-based conventional cell separation methods. Adapted with changes[108].

	Method	Principle	Advantages	Disadvantages
Label-free	Adherence	Selective adherence	Low cost, Simple process, High-throughput	Low purity, Varying/low yield
	Culture	Selective growth		
	Selective lysis	Change in osmolarity		
	Filtration	Size		
	Centrifugation	Density		
Label-based	Immunodensity cell isolation MACS FACS	Surface antigen	Sensitivity, High purity, High yield	Complexity and availability of labeling, High cost, Labeling might change cell properties

Label-free separation techniques

Different cell types and populations can be separated based on biophysical markers such as size, shape, deformability, compressibility, adhesion, and density. Based on differences in these biophysical properties heterogenous biofluids or cell mixtures can be fractionated and separated in a label-free manner. Commonly, label-free techniques allow for high throughput, involve simple processing, and are accessible at relatively low costs.

Cell adherence and culture

One of the simpler cell separation or isolation methods based on cell type-specific properties are cell adherence and selective propagation in culture. Hereby, cells are isolated from primary tissues, such as blood or bone marrow, with or without digestion and pre-purification. Specific cell types can be enriched based on their ability to adhere to plastic, such as stromal cells[51], macrophages, or fibroblasts, while non-adherent or non-proliferating cells are removed.

It is also possible to enrich cells based on their selective growth in cell-specific media. For example, hematopoietic stem cells can be selectively expanded from bone marrow

samples through long-term culture[109,110]. These methods are relatively easy to apply but usually lead to compromised purity, they can be time-intensive, and properties of expanded cells might change.

Selective cell lysis

A commonly used method to enrich and purify leukocytes from whole blood or bone marrow is selective RBC lysis. This method is based on differences in the composition, thus the properties of leukocytes and RBCs. Whole blood is diluted with solutions that lead to a swelling of RBCs and finally their rupture, caused by changes in osmolarity. This can be achieved by simply adding water, which however also affects WBCs relatively fast, or by an iso-osmotic solution containing ammonium chloride mainly affecting RBCs.

Lysing solutions can be easily prepared or bought relatively cheaply. However, extensive washing steps are necessary to remove the reagents and resulting RBC debris, WBC loss can occur, or cell functions may be affected.[111-113]

Filtration

Cell suspensions can be separated based on size and deformability differences by passing them through a filter with uniform microscale pores or meshes of a certain cut-off size. While smaller cells simply flow through the openings larger cells are collected and concentrated on the filter. Filtration is an inexpensive, simple method, often used as a pre-enrichment step for further purification, for example in stem cell isolations from digested tissues[114].

Filtration is often employed to obtain single-cell suspensions by removing cell aggregates or large particles, *e.g.* before fluorescence activated cell sorting (FACS). Prior to blood transfusion, leukocytes are routinely reduced (removal of ~99.9%) by filtration combined with charge-based adhesion, to reduce post-transfusion infection risks[115-119]. The use of filters (*e.g.* made of polycarbonate) has also been investigated for the isolation of CTCs, as well as CTC clusters as they are often larger than blood cells, allowing to further characterize and analyze the cells[120,121].

However, filtration might lead to unwanted cell losses or bias in collected CTCs, as not all CTCs are necessarily larger than blood components.[122] Apart from being prone to clogging and cell losses, it can also be difficult to decide on the right cut-off size for cell separations, as there might be size or deformability variations of different cell types. Moreover, there is usually a trade-off between the recovery and purity of the target cells.

Centrifugation

Particles or cells that are denser than the surrounding medium will eventually sediment against a barrier of equal or higher density. The sedimentation rate is proportional to the difference in the particle's density relative to the surrounding medium as well as its size. This phenomenon can be observed solely based on gravity when whole blood fractionates

in a test tube over time. RBCs sink to the bottom leaving RBC-free plasma as the top layer.[123]

This process can be accelerated by centrifugal forces (Figure 4A). Centrifugation is one of the most extensively used separation methods in laboratories and is also routinely used in the clinic as it is relatively simple, inexpensive, and can process large numbers of cells. Centrifugal forces can be used for fractionation, concentration, and washing of cells in suspensions. Altering centrifugation time or speed can be used to selectively control cell types together with the degree of packing in the bottom layer. For example, platelets can be removed from the plasma or retained, while less dense lymphocytes and monocytes can accumulate on top of the denser granulocytes.[124]

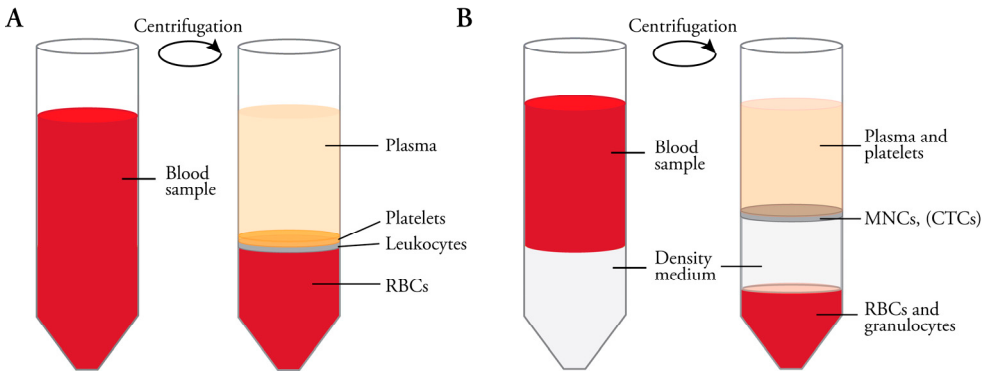

Figure 4: Schematic illustration of (A) centrifugation and (B) density gradient centrifugation of whole blood.

A sequence of centrifugation steps, also called *differential centrifugation,* can be applied to fractionate the same sample further *e.g.* to purify the cell type of interest, which can be achieved by increasing centrifugal force or time. This principle is commonly used in *e.g.* platelet preparations[124,125]. However, centrifugation may have low resolution, comprised purity, and target cells can be lost, especially when samples with low cell numbers or volumes are processed.

The separation resolution, as well as the efficiency, can be improved by *density gradient centrifugation*[126]. Here, a cell sample is layered over a graduated separation medium, such as Ficoll[127], Percoll[128], or other gradient media (*e.g.* based on polysaccharides, silica particles, or iodinated gradients). During subsequent centrifugation, a density gradient is formed in which cells move to their density equilibrium position at the isopycnic point (Figure 4B).

Density centrifugation is widely used for mononuclear cell (MNC) isolation from whole blood, PBPC products, and bone marrow samples[129]. MNCs are enriched in the thin layer between the density medium and platelet-rich plasma, enabling their collection. In a clinical setting, bone marrow harvests are usually separated by manual

or automated centrifugation to obtain MNC enriched buffy coats. In HSCT graft processing, PBPC products are often subjected to repeated low speed centrifugation for platelet removal and plasma extraction. Leading to MNC recoveries of about 75-90%.[127,130-132]

Density centrifugation is the most routinely used cell separation method in the clinic and offers large-scale cell processing. However, cell purity and recovery are largely dependent on the post-centrifugation cell collection, and require a sufficient size as well as density difference between the cell types[126]. Moreover, undesired cells with similar densities, such as CTCs, are simultaneously collected in the MNC fraction (Figure 4B)[133,134]. Centrifugation may also activate certain cell types, affect the viability or function of the processed cells[135-141].

Label-based separation techniques

Highly selective cell separation and sorting, aiming at high target cell purity, can be achieved by methods targeting specific biochemical markers or cellular components of different cell types. Typically, one or a few antibodies specific against membrane integrated surface proteins (antigens) are selected to specifically bind to the target cells for enrichment (positive selection) or to non-target cells for depletion (negative selection). This method allows to separate cell (sub)populations with similar biophysical properties which otherwise would not be separable with label-free methods.

However, labeling comes with some disadvantages. First, a specific antibody or set of antibodies needs to be identified and commercially available which limits possible separation targets. Moreover, it needs to be ensured that non-target cells do not express the selection marker, which can be challenging, *e.g.* for patient CTCs[142,143]. Furthermore, labeling with fluorescent molecules, antibodies, or microbeads may affect cell functions or fate which can affect downstream analysis and applicability, as well as the efficacy of therapeutics. Labeling processes are often labor-intense, costly, time-consuming, and usually require trained personnel.[108]

Immunodensity cell isolation

Cell types of interest can be enriched by depleting other cell types from the sample. A method commercialized by STEMCELL Technologies, RosetteSep™, is based on affinity-mediated cell aggregation of nontarget cells with RBCs, leading to denser aggregates or immuno-rosettes, which are removed in subsequent density gradient centrifugation[144,145]. For example, rare cell populations such as stem cells or CTCs can be enriched by negative depletion, whereby the cells of interest remain unaltered.

This method is fast and easy to implement into the cell isolation workflow as a pre-enrichment step. Cell preparation can be combined with positive selection methods or cells of interest may be further isolated by FACS, as commonly used *e.g.* for stem cell isolation from bone marrow or cord blood. The method is fast and does not require extra equipment but is relatively costly.

Magnetic activated cell sorting (MACS)

Magnetic sorting was developed by Miltenyi Biotech and registered as the trademark MACS[146-148]. MACS is based on the affinity-based binding of antibody-conjugated magnetic beads to cells which can be retained in a magnetic field, while non-labeled cells pass through the separation column (Figure 5A). This method is extensively employed in research and clinical applications, as it offers high throughput combined with reasonable purity.

MACS is a well-established technology and magnetic particles are available in many conjugations. Even biodegradable magnetic microbeads (~50 nm size) exist, ensuring no interference of attached particles in downstream research or clinical applications.[146] The magnetic beads can be either bound to the target cell population, *e.g.* in $CD34^+$ HSC enrichment, as utilized in stem cell transplantations (positive selection)[149-152], or bound to non-target cells for selective cell depletion (negative selection), *e.g.* used to remove CTCs[148,153] or other unwanted cells (such as $CD3^+$ cells) from the cell product[154]. MACS is also used to purify specific hematopoietic cell populations from whole blood, PBPCs or bone marrow samples.[150,155,156]

MACS is the gold standard in several clinical as well as research applications[157] but relies on a specific cell marker, mostly only one surface antigen is targeted per separation. However, MACS may influence the processed cells and can impact cell viability, proliferation, phenotype, activity or function.[158,159]

Fluorescence activated cell sorting (FACS)

Flow cytometry is one of the most widely used cell sorting and analysis techniques. FACS was already described in 1972[160]. Cells or particles are identified based on their size, shape, light scatter properties, internalization of fluorescent dyes, differential expression of specific markers, the intensity of fluorochrome-labeled antibodies bound to the surface or internal structures of the cells. As flow cytometry analyses and classifies single cells in suspensions by multiparameter optical measurements, even rare cells can be identified and purified based on their specific light scatter or surface marker profile. This is realized by the hydrodynamic focusing of cells in a narrow stream in a way that one cell at a time passes the optical interrogation points in a flow cell. Scattered light and fluorescent signals generated by the different lasers are then detected with multiple detectors, and a specific signature is created for each cell passing through the flow cell. After the analysis point, the single cell stream breaks up into airborne droplets. A charge is assigned to each droplet based on the individual information transformed into a sorting decision. Droplets are then diverted into separate collection tubes by passing through an electrostatic deflection system (Figure 5B).[161,162]

FACS machines offer event rates of ~100.000 events/sec (dependent on the machine and application) and as opposed to MACS, simultaneous multiparameter sorting of heterogeneous cell samples is possible. Although FACS provides high purity, throughput is sufficient for clinical cell analysis but not for the sorting of therapeutic

cell batches. Moreover, cells can be damaged and recovery lowered during the sorting process due to increased shear stress[162].

Equipment, material, maintenance, as well as reagent costs are high. Furthermore, using the FACS instrumentation requires sufficient training and can be time-consuming depending on the applications. Therefore, FACS is often combined with pre-enrichment steps such as density gradient centrifugation which can be additionally followed by MACS, especially for rare cell isolation.

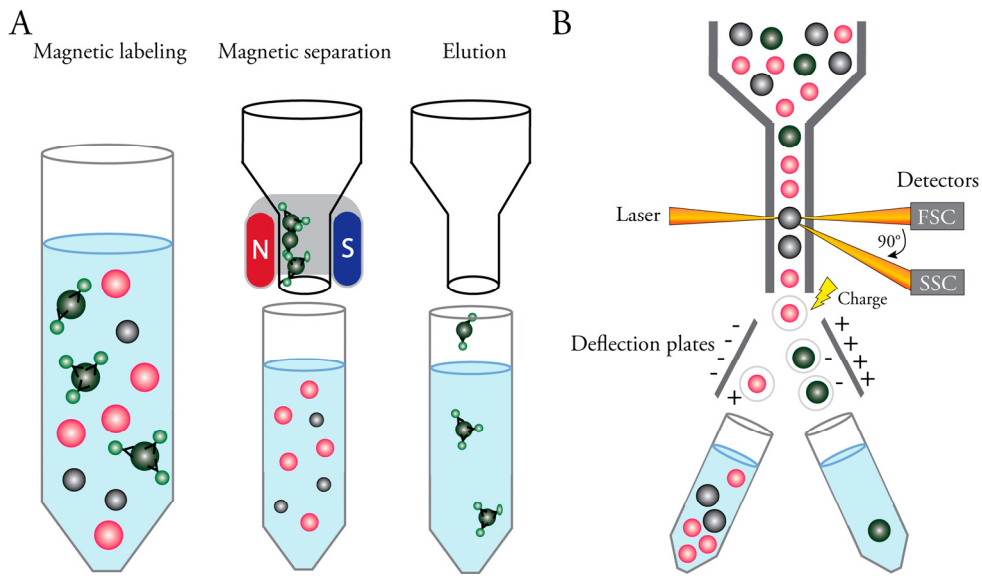

Figure 5: Schematic illustration of magnetic activated cell sorting (A) and fluorescence activated cell sorting (B).

Microfluidic cell separation methods

Microfluidics is an emerging technology that offers great capabilities to control fluids in confined volumes. The deterministic behavior of fluids in microchannels (further explained under "Acoustofluidics" pp. 29) combined with the possibility to add external force fields provide broad possibilities for precise manipulation of fluids with suspended particles and can be categorized in passive and active methods. In contrast to many conventional methods, microfluidic cell handling achieves precise label-free separation of particles based on differences in their intrinsic biophysical properties, such as size, density, stiffness, deformability, compressibility, shape, dielectric, magnetic, or acoustic properties.[163,164]

The research field has been extensively explored for many clinically relevant applications, such as blood and plasma separations, *e.g.* for blood fractionation, isolation of CTCs, bacteria, viruses, vesicles, generation of cell-free plasma, as well as some applications in BM and stem cells separations. Applications of microfluidic cell

manipulation methods have been extensively reviewed.[108,163-167] Principles of selected relevant microfluidic cell separation methods are described below (Table 3).

Table 3: Overview of selected microfluidic separation methods.

	Separation method	Separation criteria
Passive	Filtration	Size, deformability, shape
	Microstructures and DLD	Size, deformability, shape or density
	Inertia	Size, deformability, shape
	Biomimetic	Intrinsic properties
Active	Dielectrophoresis	Polarizability, size
	Magnetophoresis	Intrinsic magnetic susceptibility
	Acoustophoresis	Size, density, compressibility

Filtration

Size exclusion is one of the simplest separation criteria which can be employed on the macro or microscale. Microfluidic methods allow for precise control of fluids and enable *filtration* based on size, shape, and deformability by integrating different obstacles such as weirs, pillars, cross-flow, or membranes. They can be used in two modes, the dead-end filtration, which is prone to clog, or as cross-flow filtration which allows for extended continuous filtration (Figure 6).

Microscale filtration has been established for apheresis and fractionation of blood components as well as rare cell isolation.[9,168-171] Filters are easy to handle, label-free, fast to use, and relatively flexible. They can be commonly utilized as a pre-enrichment step. However, filters are prone to clogging when handling large cell numbers, as for macroscale filters choosing the right cut-off size remains challenging.

Cell size distributions vary and overlap; more deformable cells might be able to squeeze through the filter openings smaller than their diameter. Increased shear stress can also lead to cell loss, activation or damage.[172-175]

Another mode of filtration in microfluidics is *hydrodynamic filtration* or fractionation. Cells can be separated due to their differential positioning in streamlines based on their size, deformability, and shape (Figure 7A). Particles can be directed to different outlets by exploiting the laminar flow regime at low Reynolds numbers combined with defined chip designs and configuration, as well as the adjustment of flowrates.[163,172,176-178]

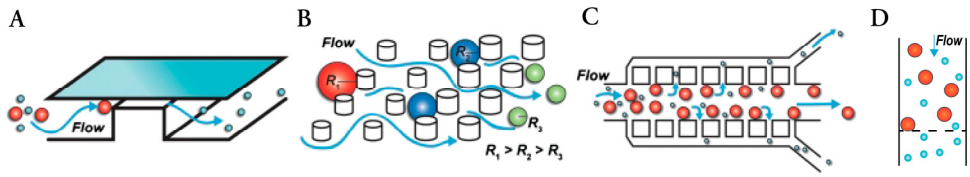

Figure 6: Microscale filter designs and separation modes. (A) Size-exclusion by wire-type filters. (B) Pillar-type filters consisting of pillar arrays with confining spacing. (C) Cross-flow filter and (D) dead-end filter. Adapted with changes and reprinted with permission from Gossett *et al.*[163], © 2010 Springer Nature.

Microstructures and deterministic lateral displacement

Microstructure protrusions, such as grooves, chevrons, herringbones or microwells, planar or lateral to the flow have been used for size-, density- and deformability-based cell separations, as well as lab-on-a-chip mixing applications (Figure 8A).

Different geometries and operation modes have been tested for isolation, enumeration, or concentration of cells from mixtures, such as CTCs from blood[179]. Microstructures can be used in combination with other separation or (affinity-)capture techniques but can be difficult to fabricate and may be prone to clogging.[180,181]

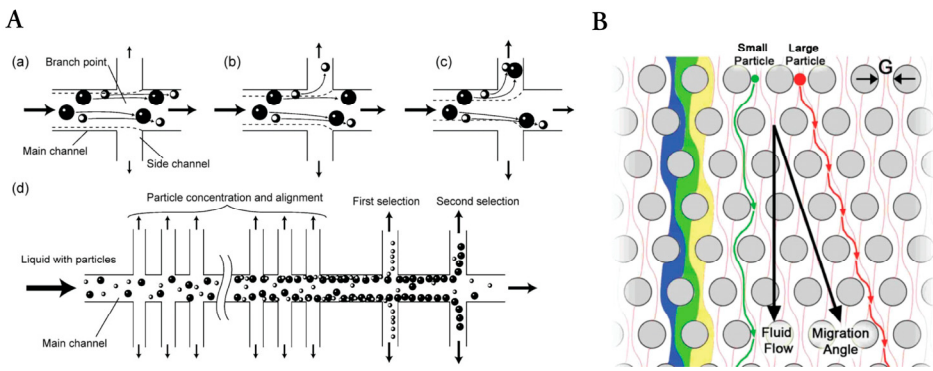

Figure 7: Hydrodynamic separation methods. (A) Principle of hydrodynamic filtration. Schematic drawings illustrating particle behavior at branch points for varying relative flow rates distributed into side channels from low (a), medium (b), and high (c). Suspended particles are infused in the main channel and become aligned and concentrated close to the walls as liquid is removed through the side channels prior to entering the section with size-selective side branches (d). Reproduced with permission from Yamada *et al.*[177], © The Royal Society of Chemistry 2005. (B) Principle of deterministic lateral displacement – DLD. Particles that flow through an array of micropillars can be separated based on size. Particles above a critical diameter will "bump" into the posts, resulting in a zigzag displacement into neighboring streamlines. Smaller particles below the critical diameter follow their original streamlines through the array. Reprinted with permission from Inglis[182], © American Institute of Physics 2009.

Deterministic lateral displacement (DLD) is a hydrodynamic technology that separates particles in continuous flow based on deformability and size, down to submicrometre levels[183]. Continuous separation of particles can be achieved by combining laminar flow with micropost arrays. Depending on the pattern of the array and the particle sizes, particles below a critical size follow their original streamline through the gaps between the posts without lateral displacement. Particles above the critical diameter "bump" laterally, crossing into neighboring streamlines, thus enabling to collect cell types following paths with different migration angles in distinct outlets at the end of the array (Figure 7B).[183,184]

DLD has been explored for numerous cell separation and processing applications, including RBCs, WBCs, platelets, CTCs, as well as stem cells.[185-191] High cell concentrations can be processed depending on the application. DLD devices show good size resolution and can be used for size measurements[192]. Sample flow rates are typically low but the separation can be upscaled by parallelization of several devices. As in filtration, clogging, shear stress and platelet activation have been reported, but also preserved viability and proliferation capability of CTCs.[189,193-195]

Biomimetic

Biomimetic microfluidic separation technologies explore the hemodynamic phenomena observed for blood in the microvasculature transferred to microfluidic systems. Blood fractionation is achieved due to the intrinsic cell properties, although the exact mechanisms are not fully understood yet. These effects include plasma skimming and leukocyte margination observed in the microvasculature and could be used to separate leukocytes directly from whole blood.

In small blood vessels, RBCs will be concentrated in the vessel center, while leukocytes are marginalized and concentrated in the plasma-rich region close to the channel walls. Furthermore, when microcapillaries split, RBCs will predominantly move to larger capillaries, also known as the bifurcation law or Zweifach-Fung effect.

These phenomena were implemented in microfluidic devices to separate malaria-infected, less deformable, RBCs from non-infected RBCs, as well as for the separation of pathogens from whole blood or to separate RBCs from WBCs and platelets (Figure 8B).[163,196-199]

Inertia

Although inertial effects are neglectable at low Reynolds numbers, certain microfluidic systems can be operated at higher flow rates with Reynolds numbers between 1-100, where inertial effects become significant. Microfluidic inertial focusing and its applications have been intensively investigated and reviewed. Inertial effects in microchannels can be used to separate particles based on size, deformability, and shape by utilizing the balance between two inertial lift forces, the wall effect lift and the shear gradient lift force. Equilibrium positions of particles can be further manipulated by changing the channel dimensions as well as geometry. In spiral or asymmetrically curved channels another inertial effect can be

observed, the Dean drag force, which was widely employed to create counter-rotating vortices in the cross-section of microchannels, also termed Dean flow (Figure 9).[200-206]

These principles have been widely implemented and explored for rare cell separations, including CTC isolation, blood fractionation, cell cycle synchronization and investigation of cultured stromal cell properties.[207-215]

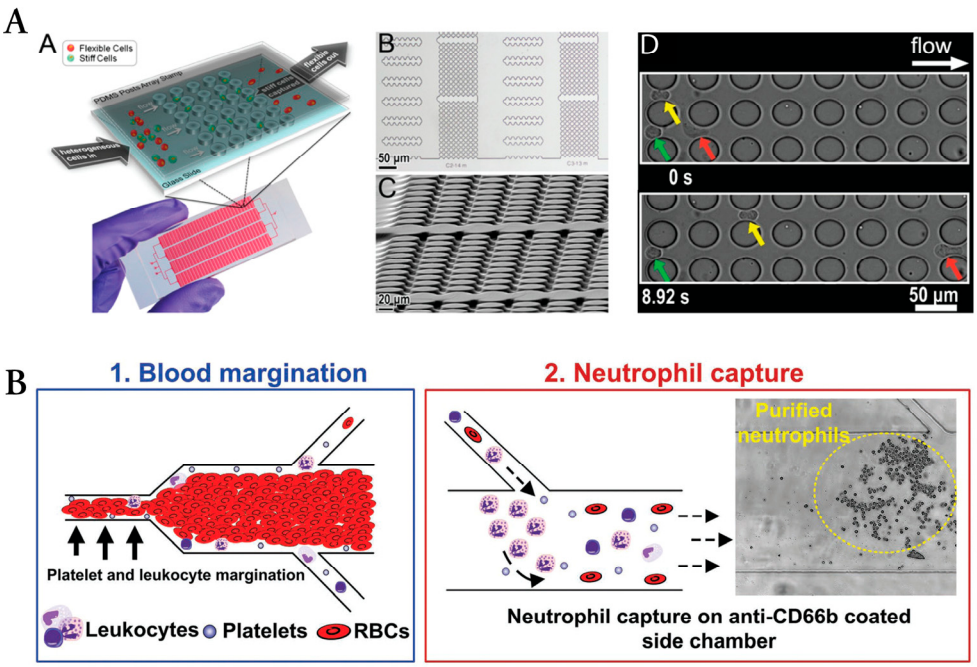

Figure 8: Microstructures and leukocyte margination for cell separations. (**A**) Microdevice for cell separation based on stiffness using artificial microbarriers and hydrodynamic force. (A):A Schematic and photograph of the device. (A):B-C Optical and scanning electron microscopic images of post arrray. (A):D Cells with different deformability (most deformable (red), less deformable (yellow), least deformable (green)) move through the post gaps shown at initial postion (0 s) and after 8.92 s. Reprinted with changes from: Zhang *et al.*[216] Microfluidics separation reveals the stem-cell-like deformability of tumor-initiating cells. Proc Natl Acad Sci U S A 109, 18707-18712 (2012)., © 2012 National Academy of Sciences USA. (**B**) Point-of-care neutrophil purification and phenotyping device based on whole blood biomimetic cell margination followed by affinity-based capture. Adapted and reprinted with permission from Tay *et al.*[217], © WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 2018.

Inertial systems are operated at high flow rates without relying on external force fields for cell separations. They provide relatively simple laboratory setups but often require considerable sample dilution. However, device designs are very application-specific and only flow rates or sample concentrations can be adjusted in a limited range to influence separation outcomes. These parameters directly influence particle focusing and particle-particle interactions. Consequently, often new applications require the specific

design of channel dimensions that need to be calculated and implemented. Nevertheless, inertial cell separation holds great potential for high throughput standard cell processing applications, such as blood fractionation.[200-206]

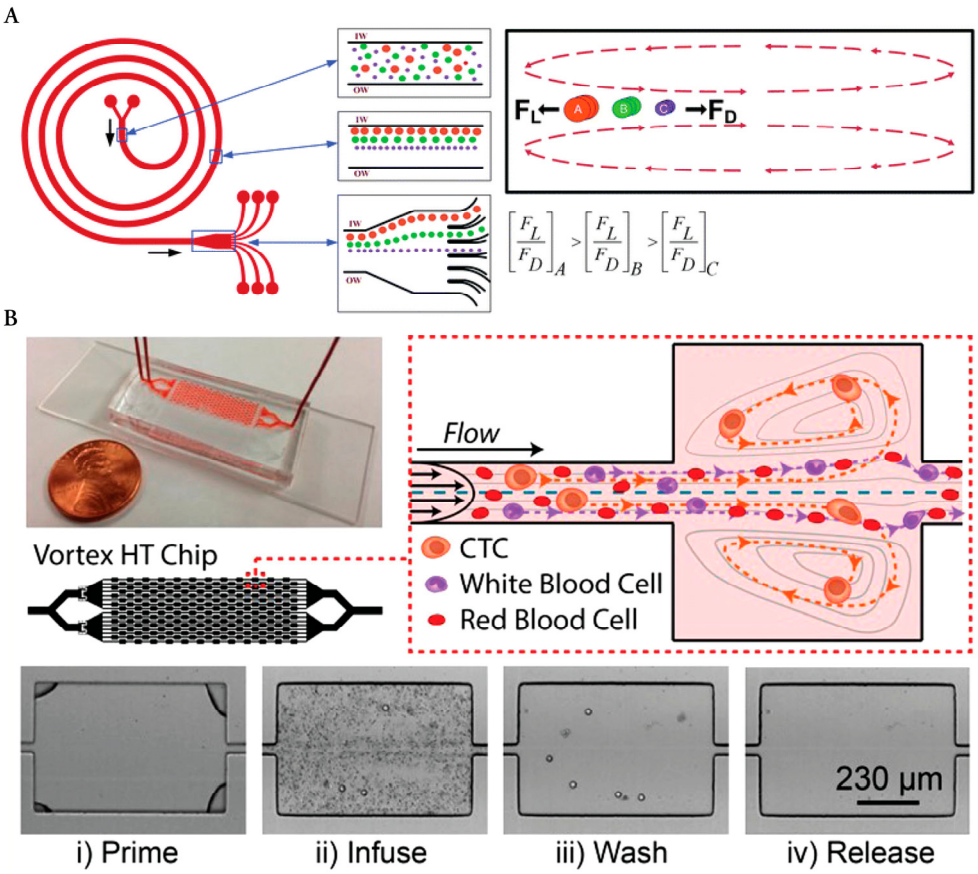

Figure 9: Inertial cell separation in spiral channels and vortices. (A) In curved channels particles equilibrate at different positions along the inner wall (IW) based on their size and the resulting balance of lift forces (F_L, directed towards IW) and Dean drag forces (F_D, directed towards the outer wall (OW)). Adapted with changes and reprinted with permission from Kuntaegowdanahalli *et al.*[218], © Royal Society of Chemistry, 2009. (B) Overview of the microfluidic Vortex HT chip for cancer cell isolation and purification. Larger cancer cells get trapped in the rectangular reservoirs at high flow rates, while other blood cells pass through or are washed away. Captured cells are released by lowering the flow rate. Reprinted with changes from Renier *et al.*[219] with permission from © Nature Publishing Group (2017).

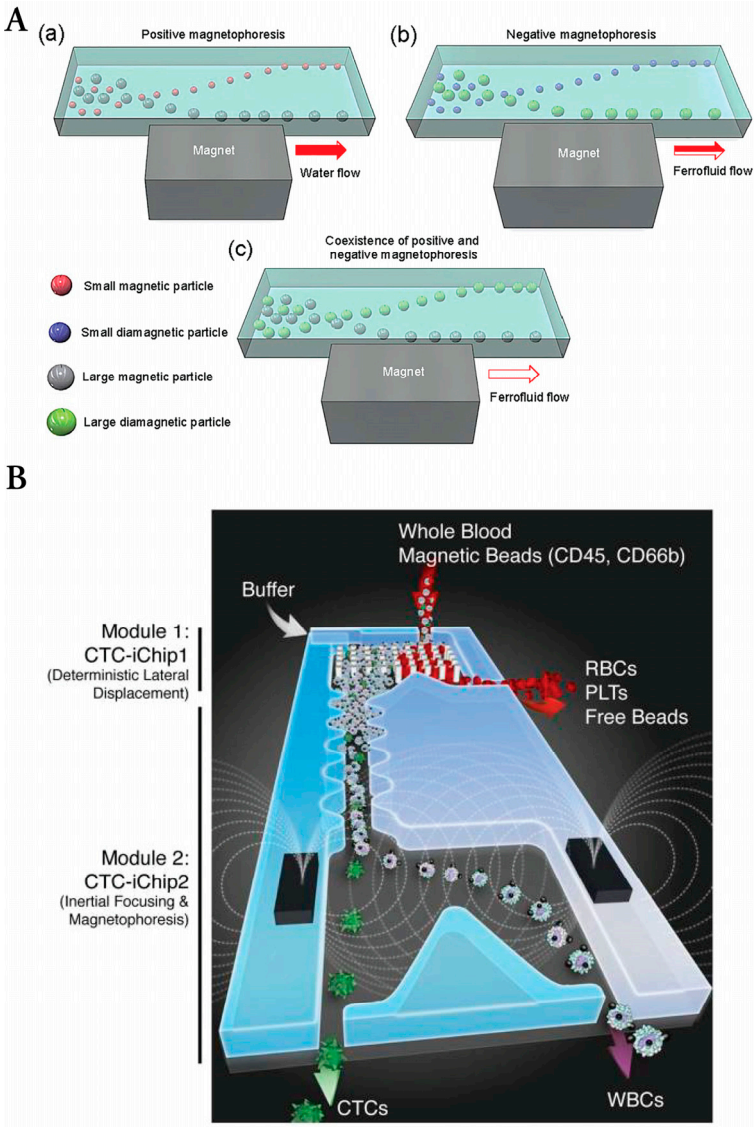

Figure 10: Magnetophoresis effects and overview of the CTC-iChip system for CTC isolation. (A) Based on particle size and magnetic susceptibility of the particles and fluid, differential magnetophoretic phenomena will be observed. Reprinted from Munaz, A., Shiddiky, M.J.A. & Nguyen, N.T. Recent advances and current challenges in magnetophoresis based micro magnetofluidics. Biomicrofluidics 12, 031501 (2018)[220], with the permission of AIP Publishing, © 2018. (B) Schematic of the CTC-iChip consisting of two modules housing three microfluidic components: First nucleated cells are isolated from whole blood by DLD, followed by an inertial focusing unit for cell alignment. In the final step bead-bound WBCs are removed from the CTCs by magnetophoresis. Reprinted by permission from Springer Nature: Nature Protocols, Karabacak *et al.*[221]: Microfluidic, marker-free isolation of circulating tumor cells from blood samples. Nat Protoc 9, 694-710 (2014), © 2014.

Magnetophoresis

Magnetophoresis can be based on the cell's intrinsic magnetic susceptibility, for example enabling label-free separation of paramagnetic RBCs (based on hemoglobin oxygenation levels), and diamagnetic WBCs in magnetic fields[222,223]. As not many cell types provide intrinsic magnetic properties, affinity-based magnetophoresis has been developed by binding magnetic particles to target cells, allowing for continuous flow separations in microdevices with integrated magnetic fields.

A differential separation is realized by deflecting magnetic particles in a non-uniform magnetic field which is directed perpendicular to the fluid flow, while non-magnetic particles are not affected (Figure 10). By using beads with different magnetic properties even multiplex separation could be realized. Implemented applications include blood, CTCs or bacteria separations, and rare cell enrichment.[158,224-229]

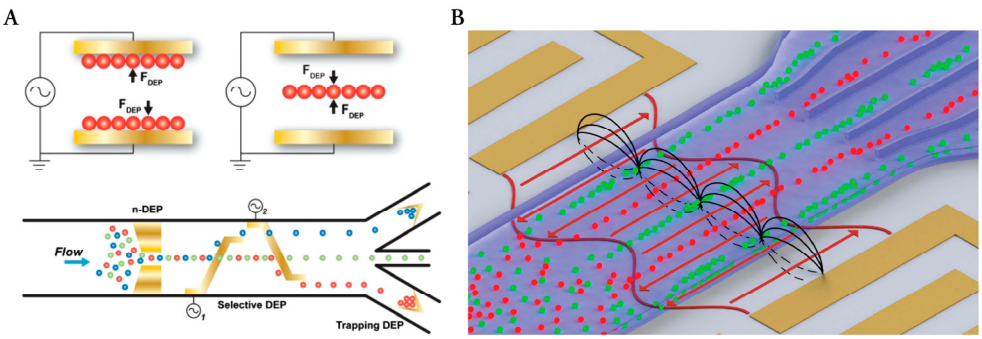

Figure 11: Principles of dielectrophoresis (DEP) and example of a surface acoustic wave (SAW)-DEP device. (A) Illustration of positive dielectrophoretic separation (pDEP, left) and negative dielectrophoretic separation (nDEP, right), affecting the positioning of the cells in the DEP-field. The lower schematic illustrates different arrangements and modes of DEP commonly used in microfluidic systems. Reprinted with permission from Gossett *et al.*[163], © 2010 Springer Nature. (B) A combination of surface acoustic wave and dielectrophoresis principles enable the separation of cells with different properties, such as viable and dead stromal cells. Black lines illustrate SAW-induced AC electric fields and lateral mechanical oscillations are indicated as red arrows. By adjusting SAW frequency and fluid conductivity it is possible for the red particles to align in areas with minimum field strength (nDEP), while the green particles align in areas of maximum field strength (pDEP). Reprinted with permission from Smith *et al.*[230], © 2017 Springer Nature.

Dielectrophoresis

Dielectrophoresis (DEP) is an active, label-free cell separation method that is based on size differences and differential dielectric properties of cell populations relative to the surrounding medium. DEP has been widely explored in microfluidics.

Particles or cells introduced into a non-uniform electric DEP force field can experience forces due to an induced or permanent dipole. The field strength can be adjusted by changing the applied voltage, AC or DC, the frequency, as well as the

electrode geometry. If a particle is more polarizable than the suspending medium it is attracted to the region with increasing field strength, also called positive dielectrophoresis (pDEP). In contrast, if the medium is higher polarizable than the particle, it will be repelled from that region of the highest field strength, also defined as negative dielectrophoresis (nDEP).

DEP can be used in continuous flow mode or as a batch process to capture cells of interest or to remove unwanted components (Figure 11A). DEP separation has been explored for several cell separation applications, including blood fractionation, CTC isolation, stem cell enrichment, and cell cycle synchronization.[231-235]

Acoustophoresis

Acoustophoresis uses ultrasound in microchannels to separate cells based on their biophysical properties, including size, density, and compressibility, in relation to the suspending medium. Acoustic devices have been developed using ultrasound as bulk acoustic waves (BAW) and surface acoustic waves (SAW).

Acoustophoresis is a label-free active separation technique that has been explored for numerous diagnostics, analytics, blood, and cell separation applications. BAW devices generally offer operation at significantly higher throughputs compared to SAW devices but rely on rigid materials to efficiently generate standing half-wavelength acoustic fields by actuating piezoceramic transducers (PZTs).

SAW devices can use standing (SSAW) or traveling (TSAW) surface acoustic waves and microchips are often fabricated in softer materials such as PDMS. Actuation is realized using interdigitated transducers (IDTs) on a rigid substrate such as lithium niobate ($LiNbO_3$). IDTs can be operated at high frequencies, allowing for precise control of particles, although at modest throughputs (Figure 11B).[236]

Hence, SAW devices have a higher potential for diagnostics and point of care applications.[237-239] Demonstrated applications include separation of viable from dead cells[230], cell washing[240], concentration[241], bacteria[242] and CTC isolation[243].

The work in this thesis is based on BAW microdevices which will be described in more detail below. Acoustophoresis using BAWs has been successfully implemented for many applications, including CTC isolation from blood preparations[244-246], cell concentration[247], washing[248,249], whole blood fractionation[250], plasmapheresis, *i.e.* removal of cellular components from blood[251,252], platelet removal from PBPCs[26], bacteria isolation[253-256], among others[239,257].

Acoustophoresis can also be combined with affinity microbeads, to selectively modify the acoustic properties of bound cell-microparticle complexes. This has been demonstrated for the separation of T-cell subpopulations with otherwise overlapping acoustic properties from PBPCs[258,259], as well as for enhanced WBC removal for the isolation of viable CTCs employing negative acoustic contrast particles[260,261].

Acoustofluidics

Acoustofluidics explores fluid dynamics in microchannels combined with the ability to manipulate particle movements using ultrasound. The field has emerged towards clinical applications in the past years and was validated for many cell handling contexts. The following section will cover some fluid dynamic phenomena of microfluidics and further describe some fundamental principles of acoustophoresis.

Laminar flow

In microfluidics, flow velocities are usually in the low Reynolds number (Re) range (<<2000), where viscosity dominates, whereas inertial effects are neglectable. Under these conditions the flow in a microchannel is laminar. The Re describes the ratio between fluid viscosity and inertial forces. It is used to predict turbulent *versus* laminar fluid behavior.

$$Re = \frac{\rho u L}{\mu}$$

Re is dependent on the average fluid velocity u, the channel dimensions L, the fluid density (ρ) as well as the dynamic viscosity (μ). In laminar flow, the velocity at a point is constant in time, moves smoothly, and predictable in space. Hence, particles follow the streamline positions in which they enter microchannels or in which they were positioned by external forces, such as acoustic wave fields.[262]

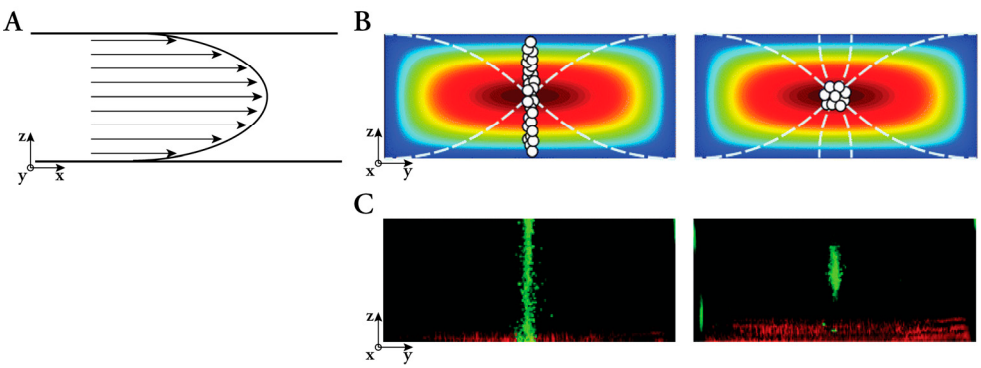

Figure 12: Flow regime and velocity profile at the microscale along with particle focusing in acoustic fields. (A) Laminar parabolic flow profile in a straight rectangular microchannel. (B) Poiseuille velocity flow profile for the cross section of a microchannel. The fluid velocity is highest in the channel center (red) and gradually decreases to zero at the walls (blue). One dimensional (left) and two-dimensional (right) particle focusing in the cross section of a microchannel as (B) schematic view, and (C) confocal images. (B), (C) Adapted from Ref.[263] with permission from © The Royal Society of Chemistry 2012.

Parabolic flow profile

In aqueous, incompressible, uniform, viscous fluids ("Newtonian fluids") at steady laminar flow in microchannels, a non-slip boundary condition at the channel walls can be assumed. This means the fluid velocity (Figure 12A, B) is at maximum in the channel center (red) and gradually approaches zero at the walls (blue), creating a parabolic flow profile, also known as Poiseuille flow. Thus, suspended particles will have differential retention times in the microchannel dependent on their proximity to the walls.[264]

Stokes' drag

One phenomenon observed for particles moving through viscous fluids induced by external force fields in a laminar flow regime is a frictional force, also known as Stokes' drag (F_{drag}). The drag force is directed opposite to the external force and the particle velocity. The force depends on the dynamic viscosity μ, the particle radius a, and the particle's velocity u. The drag force can dominate over the external force for small particles and is described as:

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

Acoustic standing wave fields

In acoustofluidics, ultrasonic standing wave fields are employed to manipulate the positioning of particles or cells in laminar flow. This has been explored in acoustophoretic particle separation, acoustic particle trapping[265], and acoustic streaming, *e.g.* in micromixers[266]. PZTs are actuated to generate ultrasound waves (>20 kHz), which can be used in a microchannel with defined, wavelength-matching dimensions to form an acoustic standing wave.

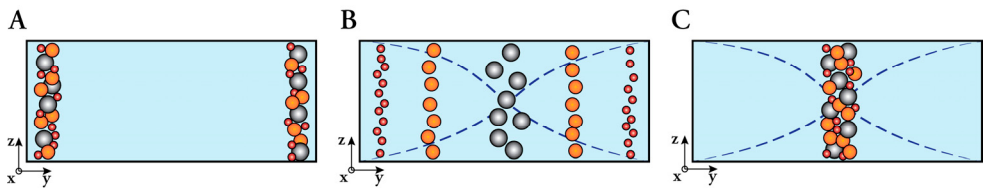

Figure 13: Particle migration in an ultrasound standing half-wavelength field. (A) Particles entering a microchannel from a side stream. (B) The radiation force in the acoustic field directs the particles towards the pressure-node in the center of the channel. Generally, larger, denser, or less compressible, *i.e.* acoustically more affected cells move faster in the field. (C) Eventually, given extended time, all particles will be focused at the pressure node (positive acoustic contrast).

Inside the channel, a force field with defined pressure nodes (amplitude minima) and pressure anti-nodes (amplitude maxima) is generated. In an ultrasound standing wave

field, different forces act on suspended particles that can result in lateral particle movement (Figure 13). These forces are the primary and the secondary acoustic radiation force, as well as the previously described Stokes' drag force.

The primary acoustic radiation force (F_{rad}) is the determining force acting on particles in cell separations and originates from the scattering of the acoustic standing waves. Depending on the particle's acoustophysical properties relative to the surrounding medium a movement will either be induced towards the pressure node or the pressure anti-node of the channel. This is also referred to as positive and negative acoustic contrast of particles. For a spherical particle (with radius $<<\lambda$) in a one-dimensional planar $\lambda/2$ acoustic field, F_{rad} can be described as follows:

$$F_y^{rad} = 4\pi\phi(\tilde{\kappa},\tilde{\rho})ka^3E_{ac}sin(2ky)$$

Whereby ϕ is the acoustic contrast factor, a is the particle radius, k is the wave number ($2\pi/\lambda$), E_{ac} is the acoustic energy density, and y is the particle's position in the wave propagation direction.

$$E_{ac} = \frac{p_a^2}{4\rho_0 c_0^2}$$

$$\phi(\tilde{\kappa},\tilde{\rho}) = \frac{1}{3}\left[\frac{5\tilde{\rho} - 2}{2\tilde{\rho} + 1} - \tilde{\kappa}\right]$$

$$\tilde{\kappa} = \frac{\kappa_p}{\kappa_0}\ ;\ \tilde{\rho} = \frac{\rho_p}{\rho_0}$$

The primary acoustic radiation force is further dependent on the pressure amplitude (p_a), the speed of sound in the suspending fluid (c_0), the isothermal compressibility of the particle (κ_p), as well as of the suspending fluid (κ_0), further the particle's (ρ_p), and the suspending fluid's (ρ_0) density. Generally, larger, denser, and less compressible particles will move faster in the acoustic force field. The direction of the movement induced by F_{rad} depends on the density and compressibility of the particle in relation to the surrounding medium. Particles with positive acoustic contrast factor move to the pressure node in the channel center, while particles with negative acoustic contrast will move towards the anti-nodes close to the channel walls.

Acoustic streaming is another phenomenon observed in acoustic standing wave fields in microchannels. It is driven by the shear stress near rigid walls in viscous acoustic boundary layers, leading to the formation of streaming rolls (Figure 14). The inner boundary layer streaming (blue), also called "Schlichting streaming"[267], is generated by the viscous dissipation of the acoustic energy creating a steady momentum flux along the rigid wall.

The orientation of the streaming rolls is from the pressure anti-node towards the pressure node of the acoustic wave field. This inner boundary layer streaming is powerful enough to drive counter rotating streaming rolls called "Rayleigh streaming"[268], within the bulk of the fluid (red).

The Rayleigh streaming induces drag forces on suspended particles. For larger particles (>2 µm), the primary acoustic radiation force commonly dominates over the acoustic streaming and particles will be focused accordingly. For smaller particles, however, the acoustic streaming will dominate over the weak acoustic radiation force. Instead of being focused, the particles will follow the streaming rolls. Thus, acoustic separation of small particles is considerably more challenging and acoustic streaming needs to be considered.[269,270]

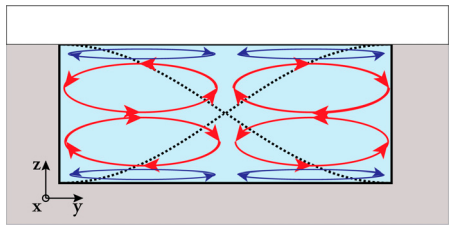

Figure 14: Schematic overview of the cross-section of a microchannel with one-dimensionally induced acoustic streaming. Inner boundary layer (Schlichting) streaming vortices (blue) caused by the attenuation of an acoustic wave in the viscous boundary layer, which is usually <1 µm for devices such as used in this thesis. The Schlichting vortices drive the outer boundary layer (Rayleigh) streaming vortices (red).

In suspensions with high particle concentrations *the secondary acoustic radiation force, or Bjerknes force,* can occur[271]. This force originates from interactions between particles in proximity and influences their relative positions. The force is usually orders of magnitude smaller than F_{rad} and is only significant at very short particle-particle distances.

The *acoustic mobility* is a parameter to assess how fast particles migrate in an acoustic standing wave field in relation to the suspending fluid. If particle and fluid properties are known it allows to predict the separability of different particle types in acoustic standing wave fields, as well as optimal fluid properties for separation.

$$Mobility_{ac} = \frac{a^2 \phi}{\eta}$$

The acoustophoretic mobility is determined by the radius a, the acoustic contrast factor ϕ of the particle, and the mediums viscosity η.

Prealignment of cells by two-dimensional focusing

Traditionally, acoustophoresis utilized the focusing of particles in one dimension[272] as illustrated in the left side of Figure 12B, and C for one node in the channel center, and two pressure nodes in the channel in Figure 15B, left. As aforementioned, the parabolic flow profile leads to different particle velocities dependent on the particle's relative location in the cross-section of the channel (Figure 12). Thus, resulting in different retention times for particles that entered the channel simultaneously at random positions, ultimately causing reduced separation efficiencies and purities of the collected cells (Figure 16A).

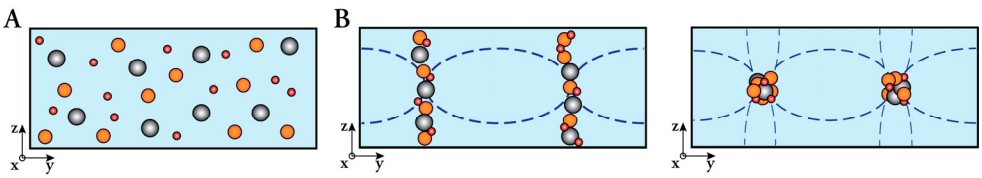

Figure 15: Acoustophoretic prealignment. (A) Particles are randomly distributed shown in a microchannel cross-section. (B) Particles are focused at the pressure-nodes within a one-dimensional ultrasonic standing wave field (left). Two-dimensional focusing is achieved by adding a second acoustic standing wave field, ensuring equal positioning of the particles in the velocity flow field of the channel (right).

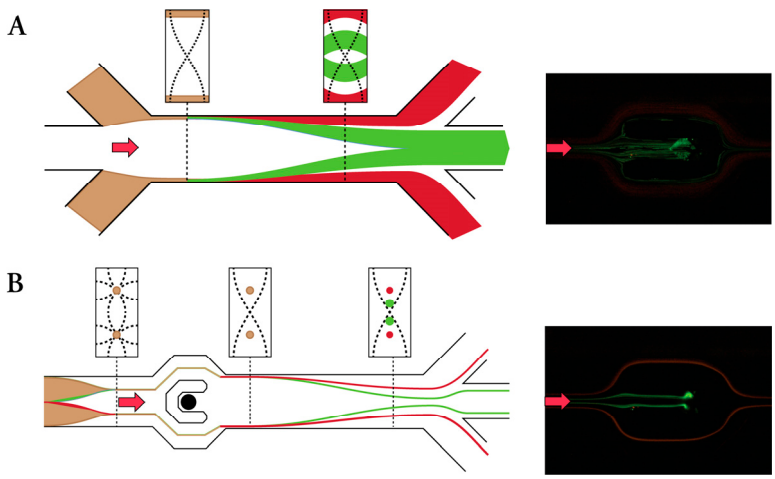

Figure 16: Schematic top view with complementary cross-section (rectangles) overview and images of the effect of acoustic prealignment on separation performance in acoustophoresis. The images on the right visualize the impact on bead separations shown for 4 and 6 µm fluorescence particles in the separation channel center outlet area for (A) one-dimensional prealignment or (B) two-dimensional prealignment. Adapted with changes by permission from Antfolk M., Laurell T. (2019), Acoustofluidic Blood Component Sample Preparation and Processing in Medical Applications. In: Tokeshi M. (eds) Applications of Microfluidic Systems in Biology and Medicine. Bioanalysis (Advanced Materials, Methods, and Devices), vol 7. Springer, Singapore © 2019.[261]

To increase the separation resolution particles can be aligned in a second dimension, as illustrated, and shown by confocal images in Figure 12B, and C (right) as well as Figure 15B. This 2D focusing ensures positioning of the particles at same flow velocity regimes (Figure 12B, right), resulting in equivalent retention times as particles enter the main separation channel.[101,246,263] This prealignment of particles was integrated in all acoustofluidic devices used throughout the articles of this thesis (Figure 16B).

Performance metrics

To assess the efficiency and compare different microfluidic separation methods some common performance metrics can be considered and are defined below as utilized in the work of this thesis. A prerequisite for cell processing is that cell function and viability are not affected, indicating the fundamental applicability of the separation methods. Especially, if the method is intended to process cells for cell therapies and transplantation preserved viability as well as function are essential. Further considerations include *separation efficiency,* or the equivalent *relative recovery* used in articles I and II, which evaluate the separation performance for different particle/cell types based on the ratio of target cells collected in the outlets.

$$Separation\ efficiency = \frac{Target\ cells_{outlet}}{Target\ cells_{all\ outlets}} \times 100\ \%$$

The *purity* of a sample or cell fraction after separation is defined as the number of target cells in a sample compared to all cells in the sample or outlet, respectively.

$$Purity = \frac{Target\ cells}{Total\ cells} \times 100\%$$

Recovery usually describes the ratio of target cells in an outlet of interest after separation compared to the number of cells of interest in the input sample.

$$Recovery = \frac{Target\ cells_{outlet}}{Target\ cells_{input}} \times 100\ \%$$

Other useful measures are *enrichment* and *depletion*, as also used in articles I and II. They compare the ratio of target cells to the whole cell population of the sample after separation in a target outlet to the same ratio of the input sample.

Moreover, *throughput* is a critical parameter for the real-life applicability of a separation method. It is usually reported as processed (sample) volume or particles per time unit.

Aims of the Thesis

The overall objective of this thesis work was to evaluate and establish acoustophoretic cell separation for processing of blood and stem cell preparations and to further develop the technology towards the application in clinically relevant contexts.

1) Investigate the applicability of label-free acoustophoretic blood and stem cell graft processing in the context of high-risk neuroblastoma therapy (Article I & II).

2) Establish label-free acoustophoretic separation to investigate subpopulation properties in cultured bone marrow stromal cells (Article III).

3) Explore acoustophoretic processing of bone marrow samples for label-free primary stromal cell enrichment (Article IV).

4) Increase throughput in multiplex acoustophoresis used in white blood cell fractionation by implementing a new microchip design and improved cooling system (Article V).

5) Evaluate the estimation of cell compressibility based on acoustophoretic separation data of various cell types and microparticles (Article VI).

Methodology

This chapter aims to give a brief overview of the main methods used throughout the work in this thesis. More detailed descriptions of the methods and techniques are included in the respective articles.

Ethical approval

All sample collections, related protocols, and procedures with human material involved in the studies herein were approved by the Swedish Ethical Review Authority and in agreement with the Declaration of Helsinki.

Acoustophoresis

Acoustophoresis describes the phenomenon that ultrasound waves can be utilized to move particles. Integrating ultrasonic standing waves in microchannels generated by piezoceramic transducers has developed into a whole new field of cell handling and manipulation with continuously increasing bioanalytical and clinical applications. This promising microfluidic technology was the principal separation method throughout the studies in this thesis and is described in more detail in the introduction part (pages 29-34).

In brief, cells or particles introduced into an ultrasonic standing wave field are subjected to the primary radiation force and will experience an induced movement dependent on their acoustophysical properties. These properties include size, density, and compressibility of the particles, also in relation to the suspending medium[273].

All microchips used throughout the work presented herein were made of silicon combined with a glass lid and had the same basic design, including two inlets, two outlets, a prealignment channel, as well as a main separation channel. There were only modifications in the channel length (articles II and V) and one additional outlet added in article V for multiplex separation of white blood cell types. It is noteworthy that the microfluidic devices used in articles I, III, IV, and VI were fabricated by anisotropic wet etching at the department of Biomedical Engineering at Lund University, while the

microchips mainly used in article II and V were fabricated by Micronit Microtechnologies (Enschede, Netherlands), using Deep Reactive Ion Etching.

Throughout the studies in this thesis, an experimental setup consisting of the same basic modules was utilized as schematically and simplified presented in Figure 17. Piezoceramic transducers resonant at frequencies of 5 MHz and 2 MHz were glued underneath the prealignment channel and the separation channel, respectively. A dual-channel function generator was employed to actuate the transducers, additionally equipped with two amplifiers, and connected to a two-channel oscilloscope to measure resulting voltages as well as frequencies over each transducer.

For temperature regulation, a PID control loop was integrated using a temperature detector attached to the 2 MHz transducer, a Peltier element placed underneath the separation channel, and a Peltier-controller (article I-IV, and VI). For improved heat transfer, the acoustofluidic devices were placed in custom made aluminum holders and positioned on an aluminum plate. For article V an air-cooling unit was implemented instead to enable improved and equal temperature distribution independent of a temperature feedback loop.

Visual control of the separation was enabled by a microscope together with a camera. The fluidics of the setup were realized by a pressure-driven system controlled by three or four flow sensors and specifically designed LabVIEW programs. All buffers used in the separation experiments were based on phosphate buffered saline (PBS) supplemented with either 2% fetal bovine serum (FBS, both Life Technologies) and ethylenediaminetetraacetic acid (EDTA, BD Biosciences), or 5% anticoagulant citrate dextrose solution A (ACDA, Terumo) and 0.1% bovine serum albumin (BSA, Sigma-Aldrich).

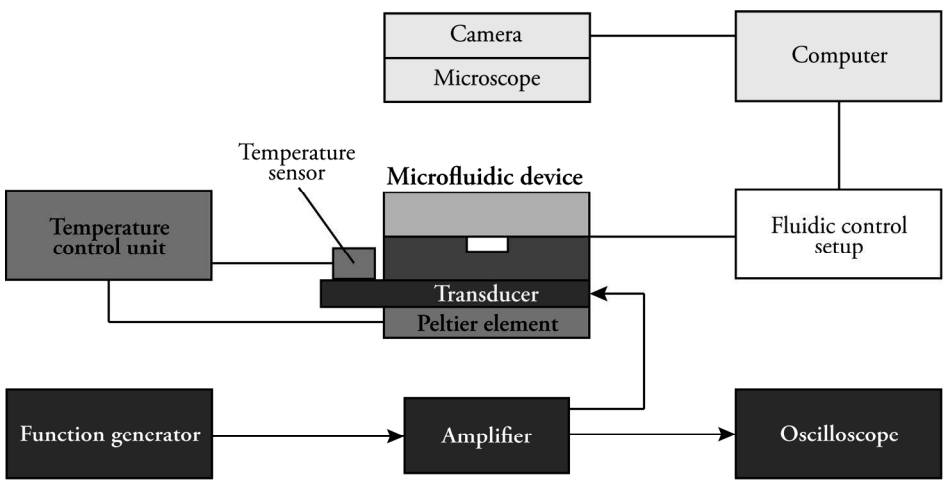

Figure 17: Simplified schematic overview of the acoustophoresis experimental setup and cross-section of the microchip utilized throughout the work in this thesis.

Flow cytometry

Flow cytometry is a very valuable method in cell biology and even clinical diagnostics because of its capability for multiparametric analysis of physical as well as biochemical characteristics of single particles and cells. Flow cytometric analysis was utilized throughout all projects of this thesis. Generally, cells were harvested, counted, washed, and blocked for non-specific binding using buffer containing PBS, supplemented with FBS or BSA, and human immunoglobulin (Gammanorm, Octapharma).

Depending on the application and project, cells were stained with directly fluorochrome-conjugated monoclonal antibodies and corresponding isotype controls. Dead cells were excluded using 7-amino-actinomycin D (7-AAD, BD Bioscience) or propidium iodide (PI, Sigma-Aldrich) staining, depending on the staining panel and gating strategy. Samples were analyzed with a FACS Canto II flow cytometer using FACSDiva software (BD Bioscience) and FlowJo v10 software (FlowJo LLC) for further analysis.

Cell size measurements

As particle or cell size is a very important parameter for acoustic separation, size distributions of various cell types were measured for the studies included in this thesis. For articles I, II, and IV-VI size measurements were performed with a Multisizer 3 COULTER COUNTER® and the complimentary Multisizer™ 3 software (Beckman Coulter). Here, the size measurements are based on impedance flow cytometry where the transient disturbance of a particle or cell passing through an electrical field is measured. The change in electrical current is proportional to the particle's volume, which is also known as the coulter principle. All measurements were performed using a 100 µm aperture tube, and electrolyte containing deionized water with 9 g/L sodium chloride (Sigma-Aldrich)[99]. In article III reported cell diameters were obtained with an automated cell counter (NucleoCounter® NC-250™, ChemoMetec A/S) that uses fluorescence staining and image-based analysis to generate data.

Human cell material

Peripheral blood samples ***(Article I, V, VI)***

Blood samples from healthy volunteers were collected in vacutainer tubes containing EDTA as anticoagulant, after informed consent was obtained. Samples were then further processed to isolate either mononuclear cells (MNCs) or white blood cell (WBC) populations.

MNCs as used in article I were purified by density gradient centrifugation with Ficoll-Paque PREMIUM (1.078 g/mL, GE Healthcare).

WBCs for article V and VI were obtained by using BD Pharm Lyse solution according to manufacturer's instructions, leading to a selective lysis of red blood cells (RBCs) in the sample.

Leukapheresis samples ***(Article I, II, VI)***

Peripheral blood progenitor cell (PBPC) samples from healthy donors and patients were obtained from leukapheresis products collected at the Clinical Apheresis Unit, Lund, Sweden, after standard mobilization treatment. The details of the collection procedure were described previously[26]. Apheresis products are highly reduced in RBC counts and enriched in WBC populations including stem and progenitor cells. In article I, PBPC samples were either used as MNC preparations isolated by density gradient centrifugation or without prior processing. In articles II and VI, PBPC samples were utilized without pre-enrichment.

Bone marrow aspirates ***(Article III, IV)***

Bone marrow (BM) samples were aspirated from the iliac crest of young healthy consenting volunteers at the Hematology Department, Lund University, Sweden.

Cell culture

Neuroblastoma cell line ***(Article I, VI)***

The human neuroblastoma cell line SH-SY5Y was purchased from the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 15% FBS, and 1% antibiotic-antimycotic solution (Sigma-Aldrich).

Neuroblastoma patient-derived xenograft (PDX) cells ***(Article II)***

Three primary neuroblastoma PDX cell lines were received from the Translational Cancer Research Laboratory (Lund University, Sweden) and grown in spherical/organoid suspension cultures as previously described[274]. PDX cells were cultured in stem cell media [3:1 DMEM/F-12 Nut Mix GlutaMAX™ (Life Technologies) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin (Biochrom), 2% B27 supplement w/o vitamin A (Life Technologies), 40 ng/mL basic fibroblast growth factor as well as 20 ng/mL epidermal growth factor (PeproTech)].

Bone marrow mesenchymal stromal cells ***(Article III, IV, VI)***

Human bone marrow mononuclear cells (BM-MNCs) were enriched from bone marrow aspirates by density gradient centrifugation with Ficoll-Paque PREMIUM (1.078 g/mL, GE Healthcare). MSCs were subsequentially isolated by selective plastic adherence through seeding of BM-MNCs at a density of 400,000 cells/cm^2 in Alpha-modified minimum essential medium with GlutaMAX™ (Thermo Fisher Scientific), supplemented with 1% antibiotic-antimycotic solution, 10% pooled human platelet lysate (pHPL, produced by the Department of Transfusion Medicine, Paracelsus Medical University, Salzburg, Austria), and heparinized with 2 U/mL preservative-free heparin (Biochrom) [pHPL medium] according to Schallmoser *et al.*[275].

Proliferation and CFU-F assays

Proliferation assays

Proliferation assays were used to investigate the effect of acoustophoretic separation and processing of the cells through the microchip on the different cell types used in the studies.

T-cell proliferation assay

T-cell proliferation capacity in article I was assessed by stimulating carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained cells with anti-CD3/anti-CD28 antibodies, as previously described[259]. Non-sorted cells and sham-sorted cells (ultrasound off) served as controls. Every other day for six days cells were harvested, and T-lymphocytes were identified by anti-CD3-APC co-staining to measure CFSE fluorescence intensity of CD3$^+$ cells with flow cytometry.

Tumor cell proliferation assay

For article I, proliferation capacity was evaluated for SH-SY5Y cells for sorted, non-sorted, and sham-sorted tumor cells. Equal starting cell numbers were then cultured for seven days and over three passages to evaluate long term impact of the different treatments. A NucleoCounter® NC-250™ was used to measure total and viable cell numbers.

PDX cell proliferation capacity was evaluated in article II for acoustically sorted, non-sorted, and sham-sorted cells. Equal cell numbers were seeded, and proliferation was evaluated after one week based on the increase in cell numbers.

MSC proliferation assay

For article III, the proliferation ability of acoustically separated cultured MSCs and controls was evaluated by plating 10,000 cells/well in 6-well plates (Sarstedt) in triplicates. After one week total cell numbers were counted with a NucleoCounter® NC-250™ to evaluate cell proliferation.

Colony forming unit fibroblast (CFU-F) assay

The CFU-F assay is a classical stromal (stem) cell assay and was used in article III for cultured MSCs as well as in article IV for BM-MNCs to assess the clonogenic capacity of respective stromal cell preparations. Cultured MSCs were seeded at 50 cells/well and BM-MNCs at 100,000 cells/well in 6-well plates in pHPL medium. Medium was changed completely on the 7th day, on the 14th day colony numbers were assessed after methanol fixation and staining with 0.1% crystal violet. Fibroblastic colonies containing ≥ 40 cells were defined as one CFU-F[51,276].

In vitro differentiation assays

In vitro differentiation assays towards the adipogenic, osteogenic, and chondrogenic lineages were performed for article II as described previously[277], to investigate if acoustic separation or the separation procedure *per se* have an impact on the differentiation capacity of cultured MSCs.

Adipogenic differentiation

MSCs were seeded into 24-well plates (4×10^4 cells/well) in triplicates and cultured in standard MSC medium [human StemMACS MSC Expansion Media (Miltenyi Biotec), supplemented with 1% antibiotic-antimycotic solution] until 100% confluence was reached. Negative control samples were continuously cultured in standard MSC medium, whereas adipogenesis was induced using AdipoDiff medium (Miltenyi Biotech). The media were exchanged every 2-3 days for two weeks, followed by formalin fixation and staining with 1% Oil Red O solution (Sigma-Aldrich).

Osteogenic differentiation

For osteogenic differentiation of MSCs, 4×10^3 cells/well were plated in triplicates in 24-well plates and after one day differentiation was induced for 21 days, with complete medium changes every 2-3 days. Induction medium consisted of standard MSC medium supplemented with 0.05 mM L-ascorbic-acid-2-phosphate, 10 mM β-glycerophosphate, 0.1 µM dexamethasone, and 1% antibiotic-antimycotic solution (all Sigma-Aldrich). Cells

were washed, ethanol fixated and stained with Alizarin Red solution (40 mM, pH 4.3, Sigma-Aldrich) to detect calcium deposits.

Chondrogenic differentiation

The chondrogenic differentiation capacity of MSCs was investigated by culturing cell pellets of 2.5×10^5 cells/pellet for 28 days. Negative controls were cultured in standard MSC medium. Induced samples were cultivated in chondrogenesis induction medium containing DMEM (high glucose) supplemented with 0.1 µM dexamethasone, 1% ITS+ culture supplements (BD Biosciences), 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate, 0.17 mM ascorbic acid, 0.35 mM L-proline (Sigma-Aldrich), as well as 0.01 µg/mL transforming growth factor (TGF)-ß3 (R&D Systems).

Following culture, cell pellets were washed, sequentially fixed with Stefanini[278], and sucrose, embedded in O.C.T Compound (Thermo Fisher Scientific), and cryopreserved at -80°C. Cryosections of 5 µm thickness were prepared, followed by staining with either hematoxylin and eosin, or 1% Alcian Blue (pH 2.5) and Fast Red solution (all Sigma-Aldrich).

Immunomodulation assay

The immunomodulatory potency of MSCs after different treatments was determined as previously described[279-281], with some modifications. Reference responder cells were CFSE-stained pooled peripheral blood mononuclear cells (pPBMCs) from ten random donors which were kindly provided by the Strunk laboratory (Experimental and Clinical Cell Therapy Institute, Paracelsus Medical University, Salzburg, Austria)[279]. MSCs were seeded in dilutions in 96-well plates to investigate a dose dependent effect and cultured overnight in pHPL medium for attachment.

The next day 3×10^5 CFSE pre-labeled PBMCs were added per well in X-VIVO™ 10 medium (Lonza) supplemented with 1% antibiotic-antimycotic solution. T-lymphocytes were stimulated with 5 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich). After 3 days, cells were harvested, labeled with CD3-APC (Clone HIT3a, BD Bioscience) and 7-AAD and analyzed with a FACS Canto II flow cytometer. Proliferation was then quantified based on the decrease of CFSE staining intensity of single, viable $CD3^+$ cells.

Cell cycle analysis

The cell cycle phase distribution of separated MSC fractions and controls reported in article II was performed using the "Two-step cell cycle analysis" assay of a NucleoCounter® NC-250™. Data were then further analyzed using FlowJo v10 software.

Quantitative Real-time PCR

Quantitative real time PCR was used for neuroblastoma cell detection in article II, based on the genes *paired-like homeobox 2B* (*PHOX2B*), *dopadecarboxylase* (*DDC*), and *tyrosine hydroxylase* (*TH*).

In article III the method was performed to further characterize the sorted cell fractions based on their gene expression of so-called stem cell genes, hematopoiesis-supporting genes, proliferation genes, and differentiation genes. The gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as housekeeping gene in all PCR experiments.

For total RNA extraction, the Arcturus™ PicoPure™ RNA Isolation Kit (Applied Biosystems, article II) and the RNeasy MicroKit (Qiagen, article III) were used. RNA concentrations along with purities were measured on a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). Subsequential cDNA synthesis was performed using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen) on a T100™ Thermal Cycler (Bio-Rad). For the RT-PCR reactions, Fast SYBR™ Green Master Mix (Applied Biosystems) and a CFX96 Real-Time PCR Detection System (Bio-Rad) were used. Reactions were always performed in triplicates.

Statistic estimation of cell compressibility

In article VI a new experimental method is presented that allows to estimate the compressibility of particles or cell populations rapidly while requiring only small sample amounts. The method uses separation data obtained by sequentially increasing the acoustic energy density during a microchip based acoustophoresis separation. Statistical analysis of the relative distributions of the cell populations recovered in the two outlets then allowed to estimate the unknown compressibility of cell populations relative to well-characterized polymer microbeads that were used for device calibration.

Present Investigation

In this chapter the articles included in the thesis are summarized shortly, presenting the motivation of the projects, some of the key findings and their implications. A more general discussion, conclusion, and outlook can be found in the following chapters. The complete articles and manuscripts are appended at the end of the thesis.

Article I

Label-free neuroblastoma cell separation from hematopoietic progenitor cell products using acoustophoresis - towards cell processing of complex biological samples

The aim of this study was to investigate the applicability of label-free acoustophoretic blood and stem cell graft processing for the possible use in high-risk neuroblastoma therapy. Neuroblastoma is an early childhood cancer with poor survival rates in high-risk patients. Currently, high-risk patients are treated with chemo- and radiotherapy, surgery, anti-GD2-therapy, combined with transplantation of autologous hematopoietic stem cells. Graft-contaminating tumor cells are correlated to inferior outcome and can contribute to relapse after transplantation.[31,282] Viable tumor cells can be removed from the graft to avoid re-transfusion, consequently decreasing the relapse risk for high-risk neuroblastoma patients. Moreover, important diagnostic and prognostic information can be extracted from collected circulating tumor cells (CTCs).

Therefore, we established a label-free acoustophoresis-based microfluidic separation technology and showed proof-of-concept evidence for diagnostic tumor cell enrichment and purging of peripheral blood progenitor cell (PBPC) products. Here, we used the SH-SY5Y neuroblastoma cell line (NBCs) spiked into blood MNC and PBPC preparations as model systems. Size differences between NBCs, blood and PBPC preparations, along with their differences in acoustic mobility were sufficient for the separation of these different cell types.

We optimized the experimental conditions and were able to efficiently enrich or remove viable neuroblastoma cells from blood MNCs as well as PBPCs using an acoustofluidic microchip (Figure 18A). It was possible to enrich NBCs with high relative recoveries of up to 60-97% from blood MNCs and PBPCs, however, leading

to compromised purities, or to collect NBCs with purities up to 90%, but with decreased recovery, respectively.

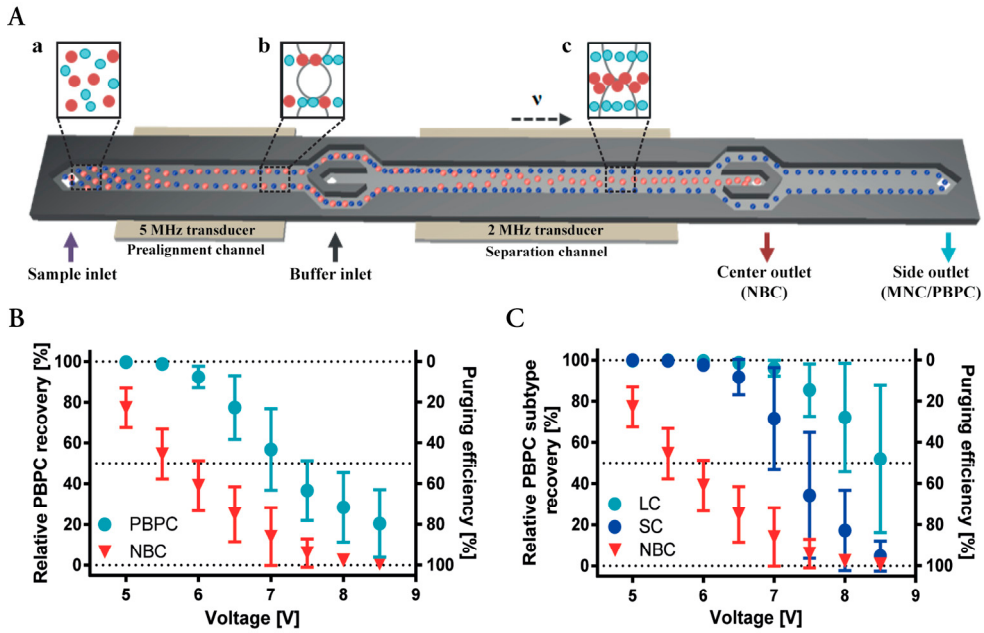

Figure 18: Acoustophoretic separation of neuroblastoma tumor cells from peripheral blood progenitor cell products. (A) Schematic overview of the acoustophoresis microchip as used in this study. Spiked neuroblastoma cells were depleted ("purged") from PBPCs (B) and PBPC subtypes (C, Lymphocytes (LC) and stem and progenitor cells (SC)) as a function of voltage [V] applied to the separation channel transducer. Graphs show the relative recovery and the respective purging efficiency in the side outlet [%].

These data suggest that it is possible to optimize the separation for two different scenarios, depending on the main objectives as well as downstream applications. Tumor cells could be either enriched with high purities for diagnostic and prognostic purposes from blood MNCs and PBPCs, or the acoustophoretic separation could be utilized to purge PBPCs aimed for transplantation with reduced contamination of viable tumor cells. In this study, substantial tumor cell removal of up to 1.5-2.3 log was achieved, while losing considerable numbers of PBPCs (over 43%). Nevertheless, it was possible to remove 60-80% of viable tumor cells while retaining over 90% of PBPCs (Figure 18B).

The PBPC product consists of various leukocyte types and the most valuable cells for transplantation are the stem and progenitor cells in this cell collection. Thus, we further evaluated the separation of NBCs from stem and progenitor cells (SC), along with lymphocytes (LC) shown in Figure 18C. These data suggest that higher separation efficiencies can be achieved when the main focus lies on the separation of NBCs from SCs and less important cell types for transplantation, such as monocytes, showing

acoustic mobility closer to the NBCs, are neglected. We also demonstrated that viability generally remained high and T-cell proliferation and long-term tumor cell proliferation were not compromised by the cell processing procedure or the acoustic separation. Preserved viability and cell functions are prerequisites for viable cell processing.

The data indicate that label-free tumor cell separation and processing of blood MNCs as well as complex cell preparations, such as PBPCs, is possible by acoustophoresis. Here, we showed proof-of-principle evidence that neuroblastoma cells can be separated from blood and stem cell preparations by label-free acoustophoresis. High relative recovery and purities could be achieved, which indicates that the technology could be further developed for label-free, gentle, continuous, and non-contact cell processing for possible future clinical applications.

Article II

Label-free separation of neuroblastoma patient-derived xenograft (PDX) cells from hematopoietic progenitor cell products by acoustophoresis

After showing proof-of-principle for the acoustophoretic separation of neuroblastoma cells from blood MNCs and PBPC samples in article I, we herein focused on the applicability of CTC separation from mobilized leukapheresis samples but spiked with neuroblastoma PDX cells. These cells retain the molecular and phenotypic characteristics of the original tumor which is why they are applied as a more realistic preclinical neuroblastoma cell model[274,283]. We further developed diagnostic tumor cell enrichment and purging from PBPC products spiked with PDX cells obtained from three different neuroblastoma patients.

We validated size differences between PBPCs and the three different PDX cell models. Furthermore, we noticed that the cell size distributions even remained unchanged over extended expansion periods in cell culture. Moreover, PDX cell proliferation was not altered by the microfluidic separation procedure, nor by their exposure to the acoustophoretic field.

We also validated the use of a longer microfluidic chip with otherwise the same design as the previously used device in article I. We achieved increased separation efficiencies and a more stable separation performance, which could be used at higher flow rates in the future. The short chip achieved relative recoveries of 92.5 ± 14.5% for PDX-1 cells, while collecting 31.5 ± 32.7% of HSCs, and 29.1 ± 18.6% of PBPCs in the center outlet, as well. For the same samples, the long chip recovered 96.8 ± 3.5% of PDX-1 cells in the center outlet but only 11.4 ± 11.5% of HSCs, and 14.7 ± 1.8% of PBPCs, respectively.

As previously, we used a throughput of 100 µL/min with 1×10^6 cells/mL sample concentration, but we were able to reduce the initial artificial tumor cell contamination to 1 in 1,000 cells and 1 in 10,000 cells by using sensitive RT-PCR as detection method

(Figure 19A). Here we confirmed the use of the genes *PHOX2B*, *TH,* and *DDC* for NB PDX cell detection in apheresis samples. Of the three different tested PDX models, two could be enriched with high efficiencies. A 2 log depletion of PDX cells from PBPCs was achieved, while retaining over 85% of stem and progenitor cells (HSCs). As previously reported, we observed better discrimination of PDX cells to HSCs compared to PBPCs. High relative recoveries of up to 98% for PDX cells were realized, while retaining over 90% of HSCs (Figure 19B).

Hence, this study provides further evidence that acoustophoretic PBPC processing could possibly be developed towards label-free, non-contact, and continuous tumor cell enrichment, as well as purging procedures for future clinical application.

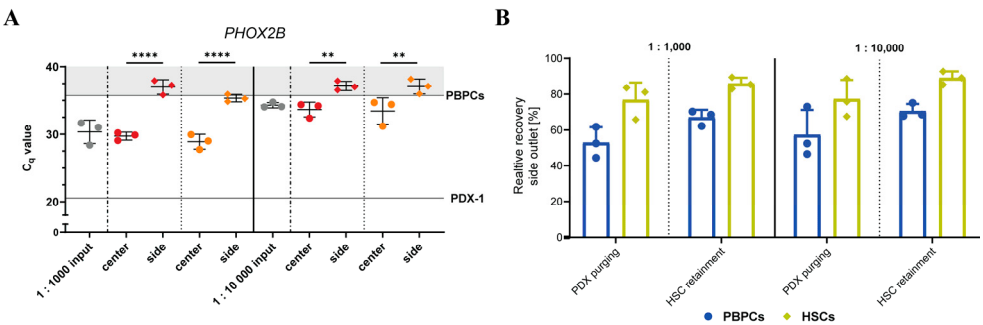

Figure 19: Sensitive tumor cell detection in separation experiments by RT-PCR with respective relative recoveries of PBPCs and HSCs. PDX-1 tumor cells were spiked into PBPCs at different ratios and separated with a sample throughput of 1×10^6 cells/mL at 100 µL/min. (A) Cq values for the neuroblastoma specific gene *PHOX2B* for input and separated samples, when aiming for purging (complete PDX removal, red symbols), or high HSCs recovery (orange symbols). (B) Respective relative recoveries [%] of PBPCs (blue) and HSCs (yellow) in the side outlet for the tested experimental conditions aiming at PDX purging or HSC retainment.

This study provided some important steps towards clinically relevant NBC separation from PBPCs, build on the knowledge gained from article I. We used three different PDX cell types, as a more clinically relevant neuroblastoma cell model, whereby two showed very similar acoustic properties, while the third was more sensitive and further overlapped with PBPC size distributions. As expected, acoustophoretic cell separation of this PDX cell model was considerably more challenging and further stresses the heterogeneity of primary neuroblastoma cells. This would need to be addressed to further develop the technology and separation procedure towards clinical applications.

The herein used throughput of 100 µL/min, equalling 1×10^5 cells/min, is at least 10 times too low to realize in-line purging during a standard leukapheresis procedure. Hence, throughput would need to be increased in the future if the technology is to be established for clinically relevant microfluidic cell processing. This could extend its real-life applicability from diagnostics and small volume applications. The microfluidic

chip used in article II could be further optimized for higher flow rates and higher sample concentrations.

A next important step in this application would be to test the acoustic separation for frozen or fresh apheresis samples from neuroblastoma patients. However, these samples are only scarcely available and precious research material. Since apheresis collections are routinely frozen, acoustic separation could be applied to thawed cell collections to remove remaining tumor cells before transplantation. On the other hand, in-line purging and enrichment of viable tumor cells during the collection procedure could be preferable to obtain a purified cell product. The isolated viable unchanged CTCs could be utilized for further characterization of the specific patient's primary tumor. This could ideally lead to adjusted personalized treatment based on the gained knowledge.

Article III

Acoustophoresis enables label-free separation of functionally different subsets of cultured bone marrow stromal cells

The aim of this study was to investigate whether label-free acoustophoresis could be used to enrich functionally different MSC populations from *in vitro* cultures. Culture-expanded mesenchymal stromal cells (MSCs), which can be isolated from various tissues, such as the bone marrow, are promising candidates for cell-based therapies. Their multipotency, immunomodulation, as well as hematopoietic support functions have led to great research efforts and their investigation in numerous clinical trials. As their frequency in tissue is very low, MSC isolation and expansion are necessary, leading to functionally promising but heterogeneous cell products. It has been reported previously that smaller cells contained in the cultured MSC product showed increased potential, *e.g.* proliferation capacity, multipotency, and colony formation[284-287].

Utilizing label-free acoustophoresis for separation of subsets from culture-expanded MSCs (passage 4) enabled to collect cell populations of significantly different size ranges, and to further investigate cell subset phenotype as well as functions (Figure 20). For example, the mean diameter of cells collected in the center outlet changed from 17.7 ± 1.8 µm at low acoustic forces to 16.6 ± 1.2 µm when separating with high acoustic forces. The respective side outlet cell diameters were lower, decreasing from 15.0 ± 1.0 µm for low acoustic forces, to 14.2 ± 0.7 µm for separation using high acoustic forces (Figure 20B). We confirmed the hypothesis that smaller sized cells, here collected in the side outlet, showed higher proliferation (+40%) and had increased clonogenic potential (2.7-fold as compared to center). Moreover, the cells collected in the side outlet showed higher expression levels of genes related to stem cell properties (*Nanog1*, *Oct4, Klf4)* and proliferation, as well as genes related to hematopoietic support functions of MSCs. For example, the expression of proliferation-related genes

was increased by 2.4-fold for *Ki-67*, and 1.7-fold for *CDK2* in side compared to center fraction cells.

We validated that acoustophoresis did not affect MSC phenotype or function. Moreover, cell viability generally remained high (87-98%) after acoustic separation and the separation procedure. All separated fractions were able to differentiate towards the adipocytic, osteogenic, and chondrogenic lineage.

Furthermore, the immunomodulatory function of MSCs remained unchanged after acoustic separation. Hence, we showed important prerequisites for the applicability of acoustic processing for cell products in cell therapies.

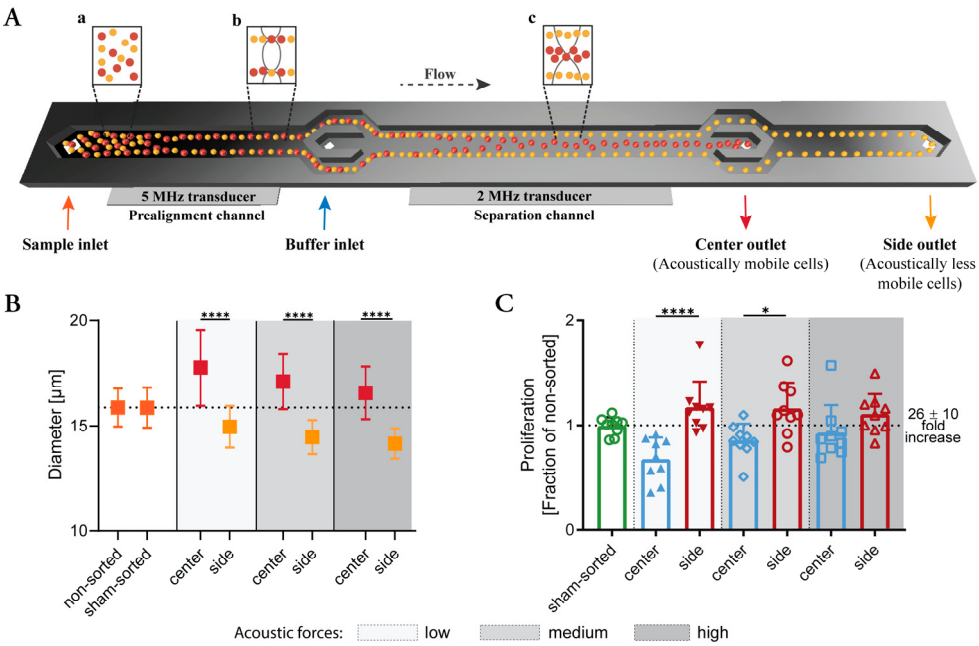

Figure 20: Acoustophoresis enables to collect subsets of cultured MSCs with distinct sizes and proliferation capacity. (A) Schematic overview of the microfluidic chip as used in this study. (B) Cultured MSCs were separated based on their distribution between the outlets resulting in the collection of cell subsets with distinct size distributions. (C) Separated cell subsets were further analyzed for their proliferation capacity and higher proliferation was observed in side outlets where smaller, acoustically less mobile cells, were collected. Outlets containing larger cells showed less proliferation.

We also established a standardization procedure that is suitable to adjust for cell size variances of cell products generated from different donors and for cells in different passages. Moreover, we observed enrichment of cells in G_0/G_1 cell cycle phase in the side fractions and an increase (up to 2.8-fold) of cells in $S/G_2/M$ phases in the center fraction compared to cells collected in the side fraction, as well as an 1.3-fold increase compared to non-sorted cells. Thus, indicating that acoustophoresis could potentially be used for cell cycle

synchronization, in agreement with previous reports on acoustophoresis and other size-based microfluidic separation methods[232,288-290].

Separating the initial cell product into fractions of even larger size differences, *i.e.* cells of smaller size ranges or exclusively larger cells, could translate into bigger functional differences. Acoustophoresis could also potentially be utilized to further purify MSC products by removing cell debris, typically present for adherend cell cultures, or by removing large senescent cells.[284,291]

The results presented in this study show the potential of label-free acoustophoresis to enrich functionally different subsets of MSCs. This can potentially be employed for further research on these MSC subsets, *e.g.* to identify high-potential cells from the heterogeneous starting material. It could also be established and further developed to produce better-defined cell products from cultured stromal cells. Hence, acoustophoretic separation could be validated as a potentially promising technology which can be a first step towards improved and standardized MSC products for research and future clinical use in improved cell therapies.

Article IV

Acoustophoretic label-free enrichment of primary stromal cells from bone marrow samples

The bone marrow contains rare skeletal progenitor cells, or mesenchymal stromal cells (MSCs), which have high potential for the development of cell-based therapies, as well as stem cell research. Because of their very low abundance, it is necessary to pre-enrich primary stromal cells from the bone marrow for various applications. Standard enrichment methods, such as FACS and MACS, require labeling of the cells which presumes the availability of a suitable and unique marker. These methods can be combined with a commonly utilized, commercially available immunodensity pre-enrichment step, which depletes hematopoietic cells (RosetteSep™). However, a label-free, gentle, low-cost, and high yield method would be preferable. It has been reported previously that MSCs are contained in the larger sized cell fraction of the bone marrow, which indicates that size-based enrichment of primary MSCs might be possible.[191,292]

This study aimed to investigate the applicability of acoustophoretic label-free enrichment of primary MSCs from BM-MNCs. We measured sufficient size differences between early-culture MSCs and BM-MNCs, and distinct acoustic mobility of these cell populations (Figure 21A). BM-MNC sizes ranged from 5-12 µm with a median of 7.2 µm in diameter, while the adherent fraction of the BM-MNCs (P0 MSCs) showed an increased median cell size of 15.2 µm. During further expansion MSC size generally increased (median 21.0 µm).

Polystyrene beads were employed as a reference to determine and standardize the choice of promising settings for acoustic separation. In a next step, we used cultured MSCs spiked

into BM-MNCs as a model system for bone marrow samples. High separation efficiencies could be achieved, *e.g.* 83.5 ± 6.1% of P1 MSCs were recovered in the center outlet, while only 16.3 ± 8.7% of BM-MNCs were collected in the center fraction, respectively.

We further investigated the enrichment of colony forming cells in separated fractions with RosetteSep™ enriched samples serving as controls. Generally, it was possible to enrich colonies in center fractions compared to side fractions (Figure 21B), and results were comparable to RosetteSep™ in a few selected separations (up to 5.5-fold direct enrichment from BM-MNCs). This indicates that further optimization of the experimental conditions is necessary to improve and standardize the acoustic enrichment of colony-forming cells. Moreover, we investigated the effect of sample concentration on CFU-F enrichment. Similar results were observed up to the tested 2×10^7 cells/mL which indicated that the throughput could be increased significantly.

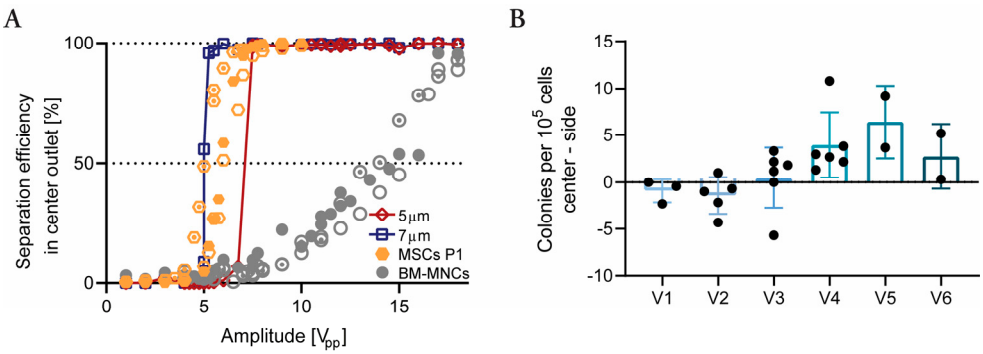

Figure 21: Cultured MSCs and BM-MNCs show distinct differences in acoustic mobility and colony forming cells could be enriched from BM samples. (A) Polystyrene beads with 5 and 7 µm diameter were mixed with P1 MSCs and BM-MNCs, respectively. Separation efficiencies were measured for varying amplitudes on the separation channel transducer. (B) Differences in colony numbers per 1×10^5 cells in center to side outlets. V1-V6 refer to experimental settings that were standardized between experiments based on defined bead separation outcomes. The amplitude on the main separation channel increases from V1-V6, whereas all other system parameters were kept constant. It was possible to enrich colonies in the center fraction compared to side fractions by label-free acoustophoresis.

The results of this study provide first evidence that primary MSCs can be enriched by label-free acoustophoresis from BM-MNCs, solely based on their distinct biophysical properties. However, isolation consistency needs to be further enhanced to reach comparable enrichment to other methods, such as RosetteSep™. Improved acoustophoretic primary MSC enrichment could then be used for better-defined cell cultures, and downstream cell analysis or sorting with label-based methods reducing the necessary amounts of reagents as well as sorting time.

Article V

Label-free separation of leukocyte subpopulations using high throughput multiplex acoustophoresis

Various clinical and research applications rely on the separation of samples composed of various cell types. WBCs (leukocytes), for example, are fundamental for the immune system. Their subpopulations are of great value for diagnostics, immune response monitoring, and the development of cellular therapies. Hence, it is of great interest to advance label-free, rapid, and cost-efficient methods for cell fractionation. Common separation methods like FACS and MACS are time-consuming, require labeling of the cells, are expensive, and trained personnel is necessary. Microfluidics have developed into a valid alternative for these conventional label-based methods, offering the possibility to explore biophysical biomarkers such as size, density, compressibility, charge, shape, deformability, or magnetic susceptibility.

This study aimed to develop multiplex acoustophoresis based on a new microfluidic device with 3 outlets. Since temperature variations during acoustofluidic separations can compromise the separation performance, the new chip design was combined with a 3D-printed chip manifold with integrated air cooling (Figure 22A).

The air-cooling unit was characterized and validated to ensure optimal thermal distribution and minimal acoustic energy losses by minimizing clamping of the chip. The effects of flow rates and air cooling on the temperature over the device were investigated, as well as the effect of prealignment voltage on separation efficiencies of beads. The proposed system allowed to simultaneously separate three different fractions at high throughputs. Its performance was evaluated using a mixture of 3, 5, and 7 µm polystyrene beads, and human leukocyte populations from whole blood preparations of viable (Figure 22B) or fixed cell samples. Moreover, the effects of particle concentrations and flow rates on separation performance were further investigated.

Simultaneous separation of the bead mixtures was possible at sample flow rates up to 500 µL/min with high efficiencies (>95.4%) and purities (>96.3%) equalling a throughput of ~2.5×10^6 beads/min. It was also possible to fractionate human viable WBCs into lymphocytes, granulocytes, and monocytes with high efficiencies of 96.8%, 99.0%, and 66.7%, respectively, along with high purities of 96.5%, 98.8%, and 71.8% at flow rates of 100 µL/min (~1×10^5 cells/min). Further increase in sample flow rates up to 300 µL/min (~3×10^5 cells/min) resulted in the fractionation of pure lymphocyte and granulocyte populations (>90% purity), but decreased monocyte purity (20.9%), due to overlapping acoustophoretic mobilities of monocytes with the other cell types.

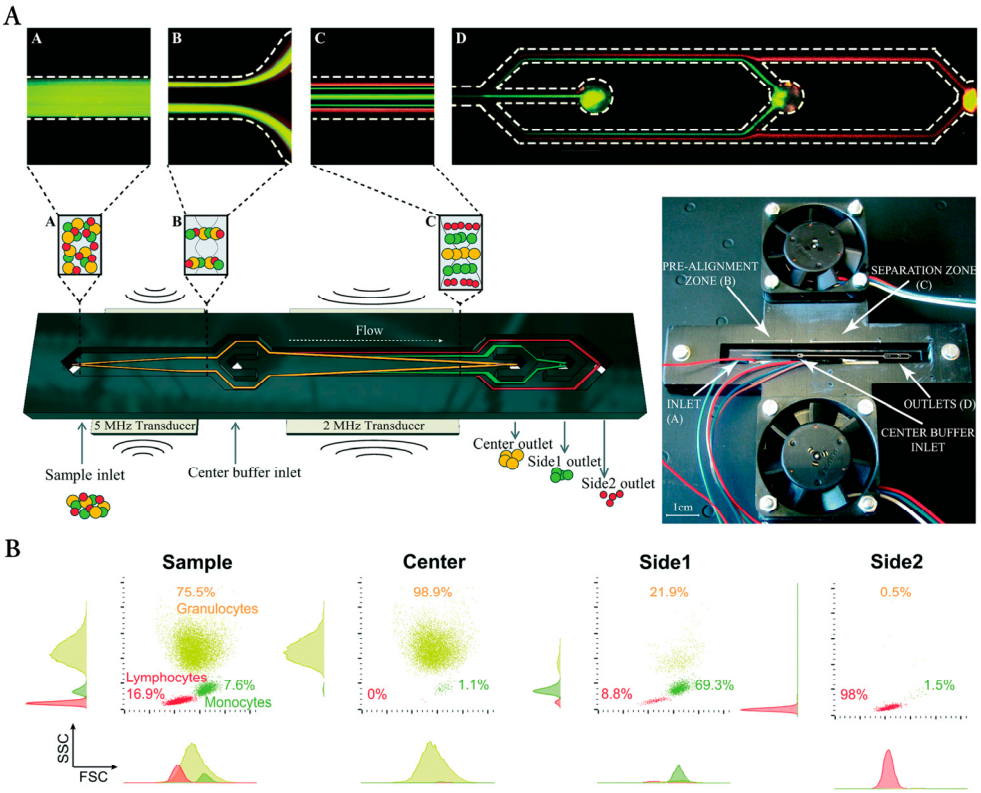

Figure 22: Multiplex acoustophoresis for label-free, high throughput separation of leukocyte subpopulations. (**A**) Schematic illustration and images of the acoustofluidic multiplex separation device: (A):A Randomly distributed cells/particles in the sample inlet (A):B are two-dimensionally pre-aligned in the acoustic field induced by the 5 MHz transducer. (A):C Finally, in the main separation channel (2 MHz transducer), the cells/particles change their lateral positions dependent on their acoustophysical properties. (A):D Beads are collected in the three different outlets, as visualized by 2, 4, and 6 µm fluorescent polystyrene beads. The photograph on the right side shows the new design of the cooling unit with integrated fans for improved thermal distribution over the whole microfluidic device. (**B**) Multiplex separation of lymphocytes, monocytes, granulocytes by acoustophoresis and their initial distribution, as well as their distribution in the different outlets after separation, presented as flow cytometry scatter plots with adjunct histograms.

Previously, temperature measurement and regulation were performed locally on the main separation transducer and channel, but experience showed that the prealignment channel can be a source of additional heating of the device. Although the aluminum holder and the mounting plate for the device supported better heat transfer, the new cooling unit ensures even thermal distribution over the whole device which results in a more stable separation. The design of the cooling unit furthermore allows for easy exchange of the microchips which eases its implementation over a wide range of applications.

The concurrent separation of bead mixtures was possible at 10 times increased flow rates compared to previously reported acoustophoresis devices. Furthermore, it was possible to significantly increase the separation efficiency as well as the purity for WBC fractionation at an over 35 times increased cell throughput, compared to a previous study using free flow acoustophoresis.[293] Consequently, the improved isothermal design of the acoustophoresis device enabled efficient, continuous, and label-free multiplexed fractionation of complex samples at a significantly increased throughput.

Article VI

Statistic estimation of cell compressibility based on acoustophoretic separation data

The possibility of separating different cell or particle types using acoustophoresis is knowingly dependent on their differential acoustophysical properties such as size, density, and compressibility, relatively to the suspending medium. However, in practice these properties are often not commonly known or found in literature, nor easily obtained. Even though size measurements can be available or are relatively easy to measure (Figure 23A), density data for cell populations are harder to obtain, and compressibility values are rarely available. Hence, it can be difficult to predict the acoustic mobility of different cell or particle types as well as the experimental conditions, such as choice of medium, necessary for optimal separation of particle mixtures.

In this study, a new method was developed that allows to measure the properties of cell and particle populations by using a theoretical approach to extract the specific compressibility of cell populations of interest from experimental separation data. Therefore, cells or particles with mostly unknown properties were mixed with reference beads of well-defined properties and separated using acoustophoresis at varying amplitudes (Figure 23B). Statistical analysis was performed for the particle counts at the two outlets (center and side) as a function of acoustic energy density (E_{ac}) during acoustic separation. Based on the distribution of the different particles, the theoretical separation performance curve (Side Stream Recovery, SSR) can be derived by a one-dimensional model. The model accounts for distributions in cell and particle properties, as well as system parameters, including hydrodynamic interactions, radiation force, acoustic streaming, and drag enhancement.

The reference beads are then used to calibrate the system and to compensate for day-to-day variations or different devices. The SSR curve is used to fit the experimental and theoretical data for the reference beads (whereby it is considered proportional to the squared voltage applied to the transducer) and results in the fitting parameter α, which is the device effectivity. Thus, the unknown compressibility of cells or particles can be estimated by fitting the experimental with the theoretical data.

The method was applied and validated by measuring the compressibility of various clinically relevant cell types, including several tumor cell lines, WBCs, together with their subpopulations (lymphocytes, monocytes, and granulocytes), $CD34^+$ stem and progenitor cells from leukapheresis samples, and cultured stromal cells from bone marrow. Hence, showing the methods applicability, rapid analysis, and the requirement of only relatively small particle numbers.

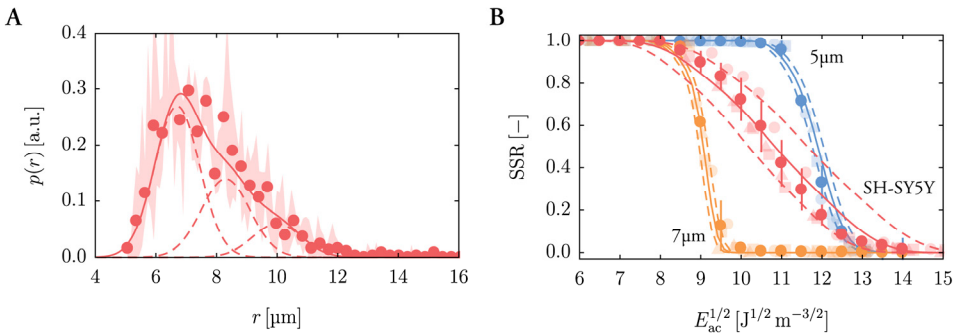

Figure 23: Cell size and acoustophoretic separation data for the statistic estimation of cell compressibility. (A) Mean and standard deviation of cell size distribution histogram for SH-SY5Y neuroblastoma cells as measured with a Coulter Counter for three independent experiments. (B) SH-SY5Y cells with unknown acoustophysical-properties were equally mixed with 5 and 7 µm polystyrene microbeads of known properties that served as reference particles for system calibration. The mixed sample was run through an acoustophoresis device at varying separation channel voltages, which are proportional to the acoustic energy density $E_{ac}^{1/2}$ in the microchannel. The dimensionless value SSR describes the side stream recovery for the separated particles. The experimental data (dots) were then used for fitting the theoretical data (lines) to extract the cell's compressibility as the fitting parameter.

The data obtained herein are comparable to a previously published macroscopic method[100], but at significantly reduced particle/cell numbers. Therefore, it is also applicable for rare cell populations such as stem cells. If size distributions are known beforehand, only minute sample amounts are required for the measurement. Otherwise, the population of interest needs to be purified for size measurements.

Widely implementing this approach would allow to collect sample specific compressibility measurements for numerous cell and particle types that can then be used to better predict the acoustophoretic separability for various sample types. This would allow to reduce trial and error approaches and instead extract unknown acoustophysical properties to predict optimal experimental conditions for different cell populations. Collecting more data and making them available would be of great interest for the microfluidic community and can possibly shorten the time for optimization spent in the lab for various cell handling applications.

General Discussion

Cell products are routinely processed in research and clinical settings for subsequent transplantation, therapeutical or analytical applications. These biological samples such as blood, stem cell products, or other cell preparations are usually complex compositions of various cell types or a heterogeneous mixture of the same cell type. The ability to successfully apply acoustophoretic separation of cells and particles generally depends on their distinct acoustophysical properties, *i.e.* differences in size, density, and compressibility, in relation to the suspending medium. The feasibility of acoustophoretic cell processing and highly discriminative separation has been demonstrated in numerous clinically relevant applications[239,257,294].

Label-free clinical-scale separation of prostate and breast cancer cells from white blood cells[244-246,256], removal of platelets from leukapheresis products[26], separation of leukocyte subtypes with and without the use of affinity-microbeads[258,259,295], as well as enrichment of bacteria from blood samples[253-256], are just some examples. Acoustophoresis has been used for separating viable cells from dead cells[247,291], and to synchronize cell cycle phases in mammalian cells[288]. Moreover, the use of acoustophoresis was realized by on-chip plasmapheresis for online diagnostics, biomarker detection[251,252], and blood or cell washing applications[248,296-298].

The technology offers many possibilities for performance and operation adjustments, not only by changing flow rates or tuning the acoustic energy put in the system, but also through modifications of the chip design. For example, changing the number of inlets or outlets or integrating several operations in one chip has been proven useful in various applications. Using one inlet reduces the complexity of the fluid system and the initial sample is not further diluted in the progress, which can be of great value in applications such as rare cell isolation[299]. If acoustic pre-focusing is implemented to increase the separation resolution, it requires the infusion of additional buffer to ensure supporting hydrodynamic focusing. Outlets can be added to remove excess fluid and obtain concentrated cells[245].

It is also possible to combine an acoustophoresis microchip with other microfluidic units as has been demonstrated for rare cell detection such as CTC or bacteria detection in blood samples[254,300]. Using multiple outlets for cell collection allows to separate complex samples as has been shown by multiplex separation of leukocytes, platelets, and erythrocytes[301], as well as, leukocyte subpopulations (article V)[295].

Furthermore, acoustophoresis is not only particle size based but also depends on the density and compressibility of particles in relation to the surrounding fluid. Thus,

medium modifications allow to separate particles that would otherwise not be separable in the acoustic field. This was demonstrated by the separation of RBCs and WBCs, as well as RBCs and platelets, which was only possible by adjusting the density of the buffer[250,301]. Lipid particles were also successfully removed from raw milk[302,303] or from erythrocytes based on their differences in acoustic properties[249].

All these examples stress the potential, including the adjustability, of the technology and point to many opportunities for new applications that are yet to be discovered or imagined. The performance of different cell separation methods can be characterized by target cell purity and recovery, depending on the application. But also, mechanical stress and impact on cell function need to be assessed, as separated cells need to maintain high viability as well as preserved biological functions. Acoustophoresis offers gentle, label-free, and continuous cell processing with high resolution separation which are important prerequisites for clinical implementation.

In microfluidics many approaches are either focused on point of care and diagnostic applications, which usually require precision for smaller sample volumes, or on methods aiming for clinical scale cell processing. For many clinical applications, such as HSCT graft processing, large scales are required with high capacity and throughput. Moreover, the processing of cells aimed for transplantation must be established and performed in closed systems or under very controlled environmental conditions, following the standards of Good Manufacturing Practice.

Although acoustophoresis is not implemented in a clinical setting yet, and further development of the technology will be required, proof of concept has been demonstrated for several clinically relevant cell processing applications.

Cell separation in diagnostics

In clinical settings, many cell separation processes are as automated as possible. However, much sample and blood handling still need to be performed manually, even in advanced laboratories. Manually processed samples and cell handling are prone to human error with subsequentially higher standard deviations in results and analysis. As diagnostic applications require high sensitivity and precision the risk of errors due to sample handling should be minimized as well as errors that may occur during sample analysis. Point-of-care devices are desirable to reduce processing and analysis times, however, further requiring the development of fast, cheap, mostly automated, easy to handle devices and procedures.

Microfluidic and acoustofluidic cell processing offer the possibility to precisely control small volumes and to standardize cell handling, thereby reducing variability while offering automation possibilities. Miniaturized point-of-care diagnostic tests and sample processing can often be realized as chip-based technologies[304]. Many common diagnostic tests involve fractionation of blood components, as described previously.

Cell enumeration can be used for health or treatment monitoring and cell removal from blood provides cell-free plasma for biochemical analysis.[9]

The field of cancer diagnostics and treatment produce the need for non-invasive sampling and analysis, also commonly referred to as liquid biopsies. Clinical applications for liquid biopsies include CTC detection, treatment response monitoring, patient screening, as well as non-invasive primary tumor cell analysis. CTCs or other tumor cell traces found in blood, such as circulating tumor DNA or vesicles, usually occur in very low numbers and concentrations, which creates the need for target enrichment, commonly compared to "finding a needle in a haystack". Especially in low stage disease, where detection is very important for early treatment, tumor material in blood and other body fluids is even lower.

The gold standard for clinical enumeration of CTCs in blood involves immunoaffinity extraction of EpCAM positive tumor cells following extensive fixation and staining protocols allowing for image-based tumor cell analysis. However, the identified tumor cells cannot be used for further molecular analysis after fixation. Thus, the method primarily serves the tumor cell detection and disease monitoring as CTC numbers correlate to tumor burden in many cancer types.[305]

To date, many microchip-based approaches have been developed for affinity-based CTC isolation, but also extensive development of label-free microchip technologies have been realized and combinations of both. One example for a combinational approach is the CTC-iChip (Figure 8B) which combines DLD, inertial focusing, and magnetophoresis for leukocyte removal, enabling processing of up to 10^7 cells/s (8 mL whole blood/h) with 97% yield of rare cells, and 3.8-log depletion of WBCs.[221,306]

Moreover, label-free spiral microfluidic devices based on inertial forces have been widely investigated for CTC isolation and demonstrated the capability of label-free CTC enrichment from clinically relevant blood volumes (usually 7.5 mL as processed with the FDA approved CellSearch system) with up to 100% cancer cell detection rates in patient samples.[208,210,215]

Acoustophoresis has also been established as label-free method to extract CTCs from blood, based on the size difference of CTC and leukocytes up to clinical-scale cell processing (5 mL blood in ~2h) with 77% viable CTC enrichment and only 0.2% WBC contamination[244].

This thesis adds further proof that acoustophoresis can be used for CTC enrichment from blood preparations as well as PBPC products demonstrated by separation of spiked neuroblastoma cell line cells and PDX cells, as further discussed under "Present Investigation". The ability to collect viable unaltered, functionally preserved CTCs enables the analysis of molecular characteristics, thus, opening the possibility to test and develop suitable therapies for the individual patient. This can further support the development of personalized treatment with minimized side effects.

Cell separation in clinical cell therapy

Therapeutic cell separation offers many opportunities for clinical applications to optimize cell transplant compositions, tailor therapy, as well as minimizing side effects by removing non-beneficial cells or contaminants. Cell populations with desired functions can be enriched for patients in clinical need, such as leukocytes by apheresis, or hematopoietic stem cells by immunomagnetic separation to obtain sufficient doses for transplantation. Moreover, regenerative treatments based on cell separation are widely explored for blood with collection and fractionation as clinical standard procedures for various disease contexts. Stromal cells from bone marrow and other tissues are widely investigated with the aim to implement their promising properties for cell therapies.

Acoustophoresis for graft processing in HSCT

In hematopoietic stem cell transplantation, the graft composition and source play an important role for the success of the transplantation, recovery of the patient, and the minimization of side effects.[307] Hence, an optimal cell transplant is sterile, has no or as few as possible contaminants that could cause side effects during or after infusion, contains sufficient numbers of stem cells to ensure a rapid hematopoietic recovery, and is ideally free of tumor cells.

To minimize transplantation complications platelets can be removed from stem cell grafts. Dykes *et al.* showed that platelets were efficiently removed by acoustophoretic cell separation (89% depletion), leading to significantly depleted amounts of activated platelets, thus decreasing the risk for platelet-leukocyte aggregation in the graft. Leukocytes were effectively recovered by 98% in the target fraction, while median viability remained constantly high (>97%) after separation.[26] In PBPC graft processing specific cell populations are often removed or isolated with MACS or FACS methods.

It has been demonstrated by Lenshof *et al.*[259] as well as Urbansky *et al.*[258] that affinity-bead mediated acoustic separation can be utilized to successfully separate cell subpopulations with otherwise overlapping acoustic properties for $CD4^+$ and $CD8^+$ lymphocytes from PBPCs, respectively. Both studies showed comparable results for acoustic and magnetic isolated target cells, whereas acoustophoresis offers continuous sample processing with preserved cell functions. Moreover, acoustophoresis provides the possibility for further development of affinity-mediated multiplex cell separation[295] by binding affinity-microbeads with different properties to multiple target populations.

The work in this thesis added proof-of-principle evidence that acoustophoresis can be utilized for tumor (cell line and PDX) cell enrichment and purging from blood preparations and PBPC products. The throughput of 6 mL/h used in Article I and II could possibly be further improved with the enhanced stabilized chip design from

article II, together with the implementation of the cooling system presented in article V. To realize acoustophoretic cell processing "in-line" with apheresis product cell collection a sample throughput of 1-2 mL/min would be required to match the collection rate of standard apheresis machines[21]. For high resolution separation as required for applications such as CTC purging, further sample dilution would be necessary for efficient acoustic separation, as discussed below and investigated in article I.

Another possible application of acoustophoresis for graft processing could be enrichment of $CD34^+$ cells, which is currently done by positive MACS selection. As discussed above, this could be realized by affinity-mediated acoustophoresis, but possibly also label-free acoustic separation as indicated by the results in articles I, II, V, and VI; HSCs and lymphocytes have similar acoustic mobilities but show differential behavior compared to monocytes, granulocytes, as well as tumor cells in the acoustic standing wave field.

Cells intended for HSCT transplantation are often frozen, stored and thawed directly before transplantation, mostly without further processing to infuse maximum amounts of viable cells. However, cell types such as granulocytes and remaining RBCs are disrupted and lysed during freezing and thawing processes. Commonly, cell debris such as granulocyte membrane fragments and enzymes, free hemoglobin or red cell stroma remain in the graft, which can lead to adverse effects post transplantation. Furthermore, as the transplant is usually not washed prior infusion, also the cryoprotectant (*e.g.* DMSO) is transfused into the patient. This can lead to side effects ranging from mild symptoms to severe life-threatening complications, thus, requiring special precautions during and after the graft infusion, especially in pediatric treatments[308]. Acoustophoretic cell processing could possibly be implemented for purification of thawed PBPC collections prior to transplantation to dilute or remove the cryoprotectant, along with dead cells and debris[247,291,298], thus, contributing to the reduction of side effects connected to the transplantation of cryopreserved cells.

Acoustophoresis offers possibilities for various gentle cell handling applications that could be utilized sequentially or implemented in one device with optimized design for the specific purpose, as successfully demonstrated for rare cell isolation as well as detection from blood[299]. In the context of graft processing and engineering these features could be further explored to offer a single microfluidic cell handling platform for multiparameter cell separation.

Acoustophoresis for BM processing and tool for regenerative medicine

The possibility to use multipotent MSCs in cell-based clinical therapies holds enormous potential for the treatment of degenerative, age-related diseases, and regenerative medicine[309]. MSCs show HSC support functions, differentiation capacity, immunosuppressive functions, tissue preservation and regenerative capabilities. They can

be easily isolated from various tissue sources and can be expanded *ex-vivo* to reach sufficient cell numbers for transplantation. These properties have led to the rapid development of investigations for their clinical use. However, MSCs isolated by standard plastic adherence are a heterogenous starting material, with additional changes of their properties during culture expansion. Surface marker profiles knowingly show dynamic expression profiles in culture. To date, no definite identification criteria for "the mesenchymal stem cell" are known. The various beneficial effects of MSCs are attributed to the heterogenous cell product without clear understanding of how differential properties translate to the specific single cell characteristics.[55]

Implementation of standard allogeneic MSC therapies, which would transform MSCs in an accessible treatment, will require strict development of uniform protocols, rigorous characterization, property and potency testing of the cell product upon release[74,310-312]. It further needs to be considered that MSC functions decrease over extended periods of *ex-vivo* expansion. This may include further lineage commitment, reduced proliferation and possibly chromosome variability, as shown for MSCs grown with FBS supplements higher than passage 4.[313]

To provide safe and well characterized cell products for transplantation, it is important to further investigate the heterogeneity of isolated MSCs. This could help to discover more defined property profiles of subpopulations in cell products which could ultimately lead to improved and application tailored MSC therapies that fulfil the guidelines for cell therapy or medical products.

To better understand the primary stromal cell population and its properties in the BM, stem cell biology research focuses on isolating these rare cells without the use of extended culture expansion[314]. Rare cells isolation protocols usually involve (immuno-)density gradient centrifugation, possibly followed by further MACS purification to decrease time along with reagent costs necessary for final FACS, which is used to obtain the population of interest at sufficient numbers and purity. Thus, leading to multistep protocols that require extended periods of cell processing, which may not only be costly but also negatively affect the target cell population and might introduce bias in the obtained cell population.

To possibly overcome some of these issues, microfluidic approaches have been investigated for enrichment of primary and early passage stromal cells. Label-free primary stromal cell enrichment based on the cells' distinct biophysical properties has been demonstrated as promising approach[191,213]. Xavier *et al.* reported higher stiffness and larger size of primary colony forming cells contained in the BM compared to most other non-colony forming BM cell types[191,292]. It has further been shown that MSC properties change considerably with *ex-vivo* cell expansion and primary stromal cell enriched cell populations, identified by $CD146^+$ or $Stro\text{-}1^+$ staining, are contained in the larger sized cell fraction of the bone marrow. The results from article IV added further proof that colony forming primary cells can be enriched from BM by label-free acoustophoretic separation, as measured by increased colony numbers in fractions enriched for larger cells. In comparison to the presented DLD device[191],

acoustophoresis offers significantly increased throughput (up to 40 times faster cell processing) as further discussed in article IV. Enrichment of primary MSCs can not only support rapid cell isolation for research purposes but could also provide a more defined starting material for *ex-vivo* expansion possibly reducing the heterogeneity of the resulting cell product.

To better understand the heterogeneity of cells generated by standard MSC expansion based on plastic adherence and subsequent cultivation, different approaches have been investigated. It has been observed early on that MSCs with higher proliferation potential and improved properties are likely contained in the smaller sized cell population of heterogenous cultured MSC products[287,315-317]. Moreover, MSCs show donor variability which commonly becomes apparent in different growth properties and differentiation capacities in culture[318]. One study reported that capacity for long-term expansion of MSCs was highly variable between different donors. After grouping MSCs from the different donors into cells with high- or low-growth capacity, it has been observed that cells with high-growth capacity were commonly smaller in size, showed greater colony forming capacity, as well as longer telomeres, thus, indicating that cell size can be an indicator for MSC function[310].

Most studies actively separating cells based on size, commonly using FACS to separate smaller sized cells from larger cells based on light scatter properties[285,286], further confirmed increased potency of small MSCs. Microfluidic cell separation devices have also been applied to fractionate heterogenous cultured cells into subsets of different size ranges. They commonly report higher multipotency and proliferation in outlets collecting smaller sized cells along with increased osteogenic or chondrogenic differentiation capacity in outlets collecting larger cells, indicating more lineage commitment of the latter[207,214,319].

In article III, included in this thesis, acoustophoresis was evaluated as another valid microfluidic technology for separation of functionally different subsets of cultured MSCs, however with the first report on pHPL supplemented cell cultures. Cell properties and viability were generally preserved. Functionally favorable cell subsets, *i.e.* showing higher proliferation, clonogenic potential, as well as gene expression profile, were collected in smaller sized fractions. Separation of cultured MSCs by acoustophoresis offers non-biased cell collection purely based on biophysical properties of the cells, which can help to understand how these properties are correlated to cell functions. Hence, possibly paving the way to more standardized, highly potent, and well characterized cell products, which is necessary as cell transplantations generally carry risks.

Consequently, standardization, validation, together with approved, tightly controlled protocols for cell product generation should be important parts of the implementation and production process. Cell medium and cultivation methods for *ex-vivo* stromal cell expansion need to be carefully chosen, as xenogenic compounds may lead to adverse effects in transplantation apart from raising ethical concerns. Xenofree alternatives are necessary for the implementation of MSC therapies in clinical

practice. Historically, MSCs have been cultured in medium supplemented with FBS and most knowledge on MSC characteristics is based on these cultures. However, studies driving research towards clinical applications implement xenofree alternatives, such as pHPL, which supports the grows of MSCs with preserved superior functions compared to standard FBS cultures[320]. The use of pHPL has been proven as a stable protein source for reliable MSC propagation. Using pHPL instead of FBS leads to cell products with improved properties, including cell proliferation, differentiation capacity, preserved immunophenotype, immunosuppressive functions, in addition to decreased immunological and infectious safety concerns.[321-323]

Consequently, the investigation of subpopulations of pHPL cultured MSCs provides the possibility to further characterize these clinically relevant cell populations and identify cell subsets with specific functional profiles. Acoustophoresis offers gentle, easily adjustable, label-free cell processing for this purpose. It is possible to obtain highly proliferative MSCs with preferable gene expression profiles. Acoustophoretic cell processing has potential for advancing research on standardized defined cell products from expanded cells as well as potential for the development of improved cellular therapies in the future.

Considerations for acoustofluidic cell processing

If microdevices shall be applied for cell handling, cells must remain unaltered by the procedure, starting by designing devices with minimum shear stress and suitable materials, as well as optimizing experimental conditions. General principles and practices for cell handling and cultivation in microsystems have been discussed in further detail in the literature[324]. Selected subjects, relevant for acoustofluidic cell processing and considered in the presented work will be discussed in the following section.

Cell property measurements and standardization

Being able to measure cell properties is an important step to reduce trial and error approaches in label-free cell separations. If the biophysical properties of target and non-target cells are known, separability as well as optimal experimental conditions could be predicted. Several methods have been proposed on the macro- and microscale, for single cell or cell population measurements[100,292,325-328]. The method established in article VI is not intended to provide single cell measurements but rather properties of the cell populations of interest. By measuring samples from several patients or donors, sample variability can be assessed. The proposed SSR method only requires low numbers of cells. It could be further utilized as basis for optimization of the separation procedure as separation data relative to reference microbeads are available right away. This enables standardization, which is necessary for clinical applications, accounting

for donor to donor variations, size or sample compositions distributions, *e.g.* differential distributions of leukocytes in blood samples, as well as differences in composition of cells dependent on metabolism and growth. Microparticles have been employed throughout all studies included in the thesis in order to calibrate the microdevices. Estimation of separation settings based on bead separation results has been further implemented in the work of article II and VI.

Biocompatibility

Generally, but also depending on the application, processing methods for biological samples, especially containing cells, need to be mild to not induce lasting stress responses, cell death, or changed cell morphology and function. Preserved cell viability and health are crucial if processed cells are intended for further use in research, analysis, or transplantation. Acoustophoresis has been proven to be a gentle and highly biocompatible method[329]. Numerous studies reported preserved cell numbers, viability, as well as functions of various cell types after acoustophoretic cell separation and acoustic exposure over extended time periods[246,247,330-333]. For example, colony-forming ability of PBPCs and lymphocyte function after acoustic sorting of PBPC products was preserved[26,258,259]. Moreover, no hemolysis of erythrocytes[296] or platelet activation as compared to standard centrifugation was observed, which is especially important for blood component processing within clinical applications[26,334]. Furthermore, the studies reported herein, showed preserved viability and functions for NB cell line as well as PDX cells, BM-MNCs and cultured MSCs.

Buffer composition and fluid properties

When exploring and designing cell processing using acoustofluidics or microfluidics in general, the chosen buffer composition must be suitable for the cells in the chosen separation procedure. For example, adding protein (such as FBS or BSA) to the buffer will reduce cell losses on plastic surfaces and further support staining with antibodies if included in the procedure. Also, anticoagulants can be added to the buffer to reduce cell aggregation over time. Adjusting the density of fluids can enable the separation of otherwise not separable cell types, but biocompatibility needs to be ensured[102,250,253,255].

On the long-term perspective towards the development of clinical applications, only approved and xeno-free reagents can be implemented to process cells intended for cell therapies. When biofluids are processed, fluid specific properties need to be considered. Blood for example is a non-Newtonian fluid due to the large number of red blood cells contained which can elongate under shear stress, leading to a change of viscosity. To achieve more aqueous Newtonian fluid behaviour (incompressible, uniform fluids with constant viscosity) whole blood is therefore often diluted or RBCs are removed before further processing in BioMEMS.

Temperature

The temperature in the system will not only have an impact on the processed sample and its components, but also on the separation performance of acoustophoretic devices as the resonance frequency is temperature sensitive (~1 kHz/°C). A temperature change of about 5°C can even result in a shift of the resonance frequency, which in consequence, can lead to a change of the resonance mode.[328] Voltage-depend temperature increase is induced by the actuation of the PZT, due to energy losses within the transducer and in coupling materials, including the fluid in the microchannel[335,336]. Thus, automated temperature control is crucial to ensure a stable performance of the acoustofluidic devices. All devices used throughout articles I, II, III, IV, and VI were temperature controlled[246,337].

An improved cooling unit was implemented in article V, providing thermal stability at elevated actuation power when separation at increased throughput was performed. Microfluidic devices, especially when not temperature controlled, are mostly operated at ambient temperature which conforms with many cell processing operations in the clinic, such as PBPC harvests[338]. Naturally, cells should not be exposed to temperatures higher than physiologically acceptable, as thermal stress and even cell death can be induced. Generally, it might be beneficial to further investigate temperature effects on cells during cell processing steps. This could especially be interesting for continuous processing of large cell volumes and cell survival could be preserved for downstream application or analysis.

Influence of particle concentrations

Higher throughput is a necessary development for the implementation of clinically relevant acoustophoresis based blood and stem cell processing for cell therapies. One way to achieve higher throughput is by implementing high cell sample concentrations; however, there are limits in acoustofluidic cell separation. With increasing particle or likewise cell concentrations hydrodynamic inter-particle interactions, along with the secondary acoustic radiation force become more prominent and will eventually lead to a decrease in separation performance.

This is dependent on the single-particle mobility, which decreases with increasing particle concentration, and a concentration dependent increase in the suspension viscosity. Moreover, at high particle concentration, particles of lower acoustophoretic mobility become hydrodynamically dragged by faster moving particles leading to a reduction in separation efficiency and fraction purity. A critical threshold for the hydrodynamic particle-particle interaction in acoustophoretic high-concentration suspensions was theoretically estimated for volume fractions greater than 0.01 (particle-to-fluid). Hence, the critical concentration is also correlated to particle size and will be considerably higher if only particle focussing is intended, *e.g.* for acoustophoretic cell or particle washing[298,339], as compared to cell separations[246,273,340].

Conclusion and Future Perspectives

Great potential lies in the cells of our body for understanding, detecting, treating, and even healing diseases. Apart from well-established bone marrow and hematopoietic cell transplantations, the use of stromal and stem cells for cell therapies is heavily investigated. Cell processing, handling, as well as separation are important tools in numerous clinical and research applications, *e.g.* in diagnostics, transfusion, cell transplantation and therapies. As the value of these processes becomes more apparent, their potential is increasingly discovered. A wide range of separation and cell processing methods exist, ranging from the macro-, to micro-, and even nanoscale. Microfluidic cell processing offers the possibility of label-free separation, based on the cells' intrinsic biophysical properties, for many applications and at high precision without the need for costly reagents or specific surface antigens.

This thesis presents work that was pursued towards evaluating and establishing acoustophoresis-based cell processing for peripheral blood and bone marrow stromal cell preparations, as well as the development of the technology towards its use in clinically relevant applications. It has been shown that acoustophoresis can be used to enrich and purge neuroblastoma cell line cells and patient-derived xenograft cells from fresh, non-fixed blood and apheresis samples. The studies presented herein provide important proof of concept that acoustophoretic graft processing can potentially be a tool during the treatment of high-risk neuroblastoma patients.

The technology was also proven applicable for the enrichment of primary colony forming cells from bone marrow samples and for the separation of functionally different subsets from cultured mesenchymal stromal cells.

A new cooling unit was presented and validated, which ensures sufficient isothermal conditions at high actuation input power, thus, stable separation for throughputs of up to 500 µL/min, which is an important advancement towards clinical scale acoustophoresis for processing of *e.g.* blood and (stem) cell preparations.

Moreover, a new method was developed that enables to statistically estimate cell or particle compressibility based on experimental data, while only requiring small sample amounts. This method allows to reduce trial and error approaches, by extracting unknown acoustophysical properties, hence, enabling to predict separability of different cell populations, and to optimize experimental conditions.

Numerous studies before demonstrated the biocompatibility of acoustophoresis for various cell types and biofluids, as well as the gentleness of acoustofluidic cell

processing. The work presented in this thesis further confirms its validity for stem cell preparations and neuroblastoma PDX cells.

The work with primary human material is subject to higher variations between samples from different patients or donors. Generally, standardization of the microchip production and assembly will be necessary to minimize device to device variations. This is especially important to enable the implementation of the technology in validated clinical protocols. Moreover, the separation process needs to be further standardized, *e.g.* by calibrating the system with beads to approximate optimal experimental settings as established herein in article II and IV, or by running test samples of the cell material aiming for a defined separation outcome, as implemented in article III.

Much effort is invested to better understand the physical mechanisms behind acoustofluidic devices, which will further improve their application, design, fabrication, and effectivity.

Some important steps were taken towards the application of acoustophoresis; however, it will still take additional work and time until acoustophoretic cell processing will be a reality in standard clinical care. Inevitably, more research will be necessary to provide acoustofluidic cell processing at clinically relevant throughputs, *i.e.* millilitres per min and in a standardized, easy to use as well as automated system. Higher throughput than presented herein could be achieved by *e.g.* parallelizing acoustofluidic devices or by developing multichannel microchips. Another consideration towards the applications of acoustophoresis for cell processing in clinical cell therapy contexts is that it will be necessary to integrate the device(s) in sterile systems. Therefore, cleaning and sterilization protocols need to be established and validated, while the devices and the materials have to fulfil the necessary requirements for medical devices. Another option could be to develop disposable microfluidic modules that can be easily exchanged between samples or patients and can be produced at reasonable costs. For example, the ongoing work on establishing acoustophoresis in plastic devices could nurture this development. All components of the setup will need to be integrated in an automated, robust, and easy to use unit that could ideally be integrated or combined with other processing steps or machines.

Moreover, great potential lies in microfluidic and acoustofluidic devices for diagnostics and prognostics, as discussed in the context of CTCs in articles I, II and in point of care devices. It is also possible to integrate several microfluidic units, for example to sequentially enrich, wash, and concentrate cells or components of interest for further analysis. It is encouraging that the usefulness of acoustic focusing is already acknowledged in a commercial flow cytometer, the Attune™ (Invitrogen), and the AcouWash system as well as the AcouPlasma modules developed by AcouSort AB. Great efforts are ongoing into further commercializing the technology for other applications. There is still a lot to learn, discover, and develop in the field of cell processing especially for clinical applications. But microfluidic and acoustofluidic cell handling open exciting opportunities for research, diagnostic and clinical applications, which will hopefully be developed into standard procedures in the future.

Popular Science Summary

The human body is a fascinating construct build of basic chemical elements, ~60% water, but also about 37.3 trillion cells, which work around the clock to keep us alive without even our conscious doing. They are the microscopic specialized building blocks of all organs, take up nutrients from the food we eat, oxygen from the air we breathe, convert them to energy, and make sure waste products, such as carbon dioxide leave the body again. An own world to keep us alive and healthy, with over hundred billion cells being renewed each day.

That is possible thanks to a few very rare cells with unique properties found in places like the bone marrow, which is a semi-solid tissue found inside bones. These so-called stem cells give rise to all more specialized cells and stay with us throughout our lives. These cells possess the exceptional feature of self-renewal, meaning that when they divide into two identical cells, one remains unchanged, while the other further develops into other cell types. Therefore, they hold great therapeutic potential, as they can restore and heal tissues.

Blood building stem cells are routinely used in stem cell transplantation, for example after chemotherapy in cancer treatment. Another stem cell type found in the bone marrow and other tissues, holds great potential for regenerative medicine as the cells can support healing and transform into several more specialized cell types such as bone, cartilage, and fat.

Blood is another important component of the body, enabling transport of cells, nutrients, oxygen, and other molecular components as well as waste products. It continuously circulates through our bodies and makes up 7-8% of the adult body weight. Blood consists of a liquid component, the so-called plasma, and different types of cells: the red blood cells, white blood cells, and platelets, which have various important functions. Red blood cells transport oxygen from and carbon dioxide to the lungs, whereas white blood cells are part of the immune system and protect our bodies from harmful bacteria, viruses, and other damaging substances. Platelets are important to stop bleedings and close wounds. Additionally, blood is also a very important indicator of body functions, due to the fact that it comes in contact with all parts if the body and its components play important roles in the immune system.

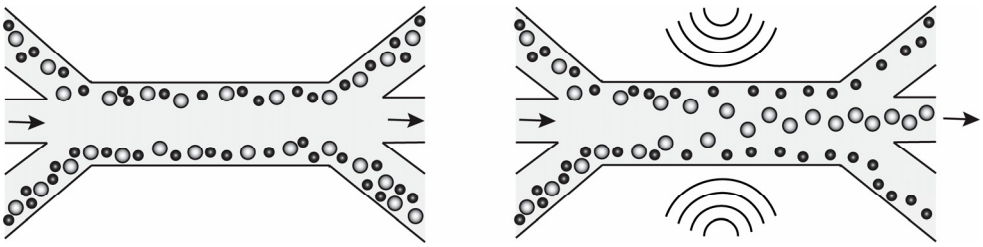

Figure 24: Basic overview of acoustophoresis. The left illustration shows cells flowing through a microchannel from the left to the right, as indicated by the arrows. Larger (light) and smaller (dark) cells enter as a mixture through the side branches, follow the flow and leave through the side branches on the opposite outlets, mixed as they entered. On the right an acoustic standing wave field is acting across the channel and the larger cells are more affected by the acoustic force, thus, move towards the channel center, while the smaller cells are less affected by the acoustic forces and stay close to the channel walls. The small cells exit through the side branches as before, while the larger cells are collected in the central branch, leading to the separation of the two cell types.

Modern medicine has come a long way, we already know a lot about how the body works, what happens if we get sick, and how to treat many diseases. However, there are still many secrets to discover. To make use of and better understand different blood or bone marrow components it is often necessary to collect certain cell types or remove others from these complex materials.

The work of this thesis investigated the usage of a microfluidic technology, called acoustophoresis, for the separation of blood and stem cell preparations. "Acoustophoresis" basically means migration (movement) by sound. In the case of the technology it refers to ultrasound which can be used to manipulate cells in microchannels. If the cells have different physical properties, such as size, density, and compressibility, they will move to either different positions or with different velocities within a standing ultrasonic wave field (Figure 24). The movement is also dependent on the properties of the surrounding medium. Using a microchannel with a width of less than half millimeter allows to precisely predict the behavior of the fluid. Cells will only change their position dependent on the forces in the acoustic field. Therefore, it offers well controllable cell handling which can be used to separate cells solely based on differences in their physical properties, as shown throughout the applications in this thesis.

First, it was demonstrated that acoustophoresis can be used to enrich circulating tumor cells from blood samples, as well as to remove them from stem cell collections. That may reduce the relapse risk of neuroblastoma patients by not infusing living tumor cells with the cell transplant. Neuroblastoma is a very lethal childhood cancer in severe cases, which accounts for about 50% of all patients. Collected tumor cells could also be used to monitor treatment and better understand the specific tumor.

Moreover, the work herein investigated how acoustophoresis can be used to separate cells from bone marrow for different applications. As stem cells are very rare, it is

necessary to further grow these cells in the laboratory to generate enough cell numbers for transplantation. During the expansion process the cells can change or lose some of their properties, whereby some cells maintain their important functions for a longer time than others. For transplantation it is very important to have well characterized cells. Acoustophoresis could be used to better understand differences in cultured stromal cells, thus, to identify and isolate cells with favorable properties.

A challenge in stem cell research is to isolate these rare cells from tissue. Herein, it was demonstrated that acoustophoresis can be used to enrich these rare primary stromal cells from fresh bone marrow samples. This is of use to obtain better-defined starting material for cell expansion in the laboratory. Moreover, acoustophoresis could be used as a first enrichment step to reduce the time it takes to further isolate these rare cells for research purposes which otherwise requires expensive or time-consuming methods.

To be able to implement acoustophoresis for stem cell processing in research and clinical applications it is especially important to increase the methods throughput. Hence, it was investigated how to upscale the acoustic cell separation. This was demonstrated by the separation of different white blood cell types from each other at shorter processing times.

It can be labor-intensive to investigate if different cell types of interest can be separated if their physical properties are unknown. Therefore, a method was developed to estimate the acoustic properties of different cell types based on separation data and statistical analysis. This is very useful to predict how different cell types will behave in an acoustic field. Hence, it can be evaluated if the cell types can be separated and what might be optimal experimental conditions. This allows to start experiments with an adjusted acoustophoresis system instead of time-consuming trial-and-error approaches.

Thus, the work of this thesis provides some new insights into to applicability of acoustophoresis for stem cell preparations. The method allows to separate cells based on their acoustic properties in a predictable, continuous, and scalable way. The technology shows great potential to be possibly implemented in several research and clinical applications.

Populärvetenskaplig sammanfattning

Människokroppen är en fascinerande konstruktion av grundläggande kemiska grundämnen och ~60% vatten, men också cirka 37,3 biljoner celler, som arbetar dygnet runt för att hålla oss vid liv utan att vi ens behöver göra något medvetet. De är de mikroskopiska specialiserade byggstenarna i alla organ. De tar upp näringsämnen från maten vi äter och syre från luften vi andas in för att sedan omvandla det till energi och slutligen se till att avfallsprodukter, som koldioxid, lämnar kroppen igen. En egen värld för att hålla oss levande och friska, med över hundra miljarder celler som förnyas varje dag.

Det är möjligt tack vare några få mycket sällsynta celler med unika egenskaper som finns på platser som bland annat benmärgen, en halvfast vävnad som finns inuti ben. Dessa så kallade stamceller stannar hos oss hela livet och ger upphov till alla andra mer specialiserade celler. Stamceller har det exceptionella inslaget av självförnyelse, vilket innebär att när de delar upp i två identiska celler förblir den ena oförändrad, medan den andra kan utvecklas vidare till andra celltyper. Eftersom de kan återställa och läka vävnader på detta sätt har de stor terapeutisk potential.

Blodbyggande stamceller används rutinmässigt vid stamcellstransplantation, till exempel efter kemoterapi vid cancerbehandling. En annan stamcellstyp som finns i benmärgen och andra vävnader har stor potential för regenerativ medicin eftersom cellerna kan stödja läkning och förvandlas till flera mer specialiserade celltyper som ben, brosk och fett.

Blod är en annan viktig del av kroppen som möjliggör transport av celler, näringsämnen, syre och andra molekylära komponenter samt avfallsprodukter. Blodet cirkulerar kontinuerligt genom våra kroppar och utgör 7-8% av den vuxna kroppsvikten. Blod består av en flytande komponent, den så kallade plasman, och tre olika cell typer: röda blodkroppar, vita blodkroppar och blodplättar, som alla har olika viktiga funktioner. Röda blodkroppar transporterar syre från och koldioxid till lungorna. Vita blodkroppar är en del av immunsystemet och skyddar våra kroppar från skadliga bakterier, virus och andra farliga ämnen. Blodplättar är viktiga för att stoppa blödningar och täta sår. Dessutom är blod också en mycket viktig indikator på kroppsfunktioner, eftersom det kommer i kontakt med alla delar av kroppen och blodets komponenter spelar viktiga roller i immunsystemet.

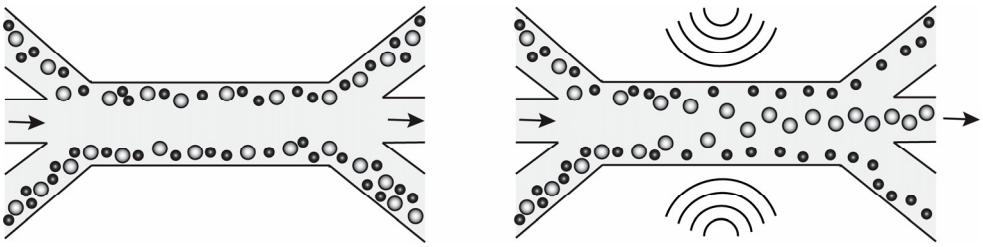

Bild 25: Grundöversikt av akustofores. Bilden till vänster visar celler som flödar genom en mikrokanal från vänster till höger, vilket indikeras med pilar. Större (ljusa) och mindre (mörka) celler kommer in blandade genom sidogrenarna, följer flödet och lämnar genom sidogrenarna via motsatta utlopp, lika blandade som när de kom in. Till höger verkar ett akustiskt stående vågfält tvärs över kanalen och de större cellerna påverkas mer av den akustiska kraften och rör sig därmed mot mitten av kanalen medan de mindre cellerna påverkas mindre av de akustiska krafterna och håller sig nära kanalväggarna. De små cellerna går ut genom sidogrenarna som tidigare, medan de större cellerna samlas i den centrala grenen, vilket leder till separationen av de två celltyperna.

Modern medicin har kommit långt. Vi vet redan mycket om hur kroppen fungerar och vad som händer under sjukdomsförlopp, samt hur man behandlar många sjukdomar. Det finns dock fortfarande många hemligheter att upptäcka. För att kunna använda och bättre förstå olika blod- eller benmärgskomponenter är det ofta nödvändigt att isolera vissa av dem eller ta bort andra celltyper från dessa komplexa material.

Den här avhandlingen undersökte användningen av en mikrofluidteknik, kallad akustofores, för att separera blod- och stamcellspreparat. ”Akustofores” betyder att flytta (migrera) med ljud. När det gäller tekniken som har använts i denna avhandling avser det ultraljud som kan användas för att manipulera celler i mikrokanaler. Om cellerna har olika fysiska egenskaper, såsom storlek, densitet och kompressibilitet, kommer de att påverkas annorlunda när de utsätts för en stående ultraljudsvåg. Beroende på cellernas egenskaper kommer de flytta sig genom vätskan med olika hastigheter eller till helt olika postioner. Förflyttningen påverkas även av den omgivande vätskans egenskaper. Med hjälp av en mikrokanal med en bredd av mindre än en halv millimeter kan man förutsäga vätskans beteende exakt. Cellerna kommer bara att ändra sin position beroende på krafterna i det akustiska fältet (Bild 25). Därför erbjuder mikrokanalen väl kontrollerbar cellhantering som kan användas för att separera celler enbart baserat på skillnader i deras fysikaliska egenskaper, vilket visas genom tillämpningarna i denna avhandling.

Först demonstrerades att akustofores kan användas för att isolera cirkulerande tumörceller från blodprover såväl som för att ta bort dem från stamcellsamlingar. Det kan minska återfallsrisken hos neuroblastompatienter genom att inte infusera tillbaka levande tumörceller med celltransplantationen. Neuroblastom är en mycket dödlig barncancer i allvarliga fall, vilket står för cirka 50% av alla fall. Samlade tumörceller kan också användas för att övervaka behandling och bättre förstå den specifika tumören.

Dessutom undersökte arbetet här hur akustofores kan användas för att separera celler från benmärg för olika tillämpningar. Eftersom stamceller är mycket sällsynta är det nödvändigt att ytterligare odla dessa celler i laboratoriet för att generera tillräckligt med cellantal för transplantationen. Under expansionsprocessen kan cellerna ändra eller förlora några av sina egenskaper, varigenom vissa celler behåller sina viktiga funktioner längre än andra. För transplantationen är det mycket viktigt att ha väl karakteriserade celler. Akustofores kan användas för att bättre förstå skillnader i odlade stromaceller för att identifiera och isolera celler med gynnsamma egenskaper.

En utmaning inom stamcellsforskningen är att isolera dessa sällsynta celler från vävnad. Här demonstrerades att akustofores kan användas för att berika dessa sällsynta primära stromaceller från färska benmärgsprover. Detta är användbart för att erhålla bättre definierat utgångsmaterial för cellutvidgning i laboratoriet. Dessutom kan akustofores användas som ett första anrikningssteg för att minska tiden det tar att ytterligare isolera dessa sällsynta celler för forskningsändamål som annars kräver dyra eller tidskrävande metoder.

För att kunna implementera akustofores för stamcellsbehandling i forskning och kliniska tillämpningar är det särskilt viktigt att öka metodens hastighet. Följaktligen undersöktes hur man kan uppskala den akustiska cellavskiljningen. Detta visades genom separering av olika vita blodkroppstyper från varandra vid kortare behandlingstider.

Det kan vara arbetsintensivt att undersöka huruvida olika celltyper av intresse kan separeras om deras fysiska egenskaper är okända. Därför utvecklades en metod för att uppskatta de akustiska egenskaperna hos olika celltyper baserat på separationsdata och statistisk analys. Detta är mycket användbart för att förutsäga hur olika celltyper kommer att bete sig i ett akustiskt fält. Därför kan det utvärderas om det är möjligt att separarera celltyperna och vad som är optimala experimentella förhållanden. Detta gör det möjligt att justera ett akustoforessystem innan ett experiment påbörjas, vilket sparar mycket tid jämfört med en rent testbaserad metod.

Således ger arbetet i denna avhandling några nya insikter i användbarheten av akustofores för stamcellspreparat. Metoden gör det möjligt att separera celler baserat på deras akustiska egenskaper på ett förutsägbart, kontinuerligt och skalbart sätt. Tekniken visar stor potential att kunna implementeras i flera forsknings- och kliniska tillämpningar.

Populärwissenschaftliche Zusammenfassung

Der menschliche Körper ist ein faszinierendes Konstrukt bestehend aus chemischen Grundelementen, ~60% Wasser, aber auch aus etwa 37,3 Billionen Zellen. Diese arbeiten rund um die Uhr, um uns am Leben zu erhalten, ohne dass wir bewusst etwas tun. Zellen sind mikroskopisch kleine Bausteine aller Organe. Sie nehmen Nährstoffe aus der Nahrung auf, sowie Sauerstoff aus der Luft, die wir atmen, und wandeln sie in Energie um. Außerdem sorgen sie dafür, dass Abfallprodukte wie Kohlendioxid den Körper wieder verlassen. Eine eigene kleine Welt, die uns am Leben und gesund hält. Über 100 Milliarden Zellen werden täglich erneuert.

Dies ist möglich dank weniger sehr seltener Zellen mit einzigartigen Eigenschaften. Sie sind in Geweben wie dem Knochenmark zu finden, einem halbfesten Gewebe, das sich innerhalb von Knochen befindet. Diese sogenannten Stammzellen sind der Ursprung aller spezialisierten Zellen und begleiten uns unser ganzes Leben. Diese Zellen besitzen die außergewöhnliche Fähigkeit zur Selbsterneuerung. Wenn sich eine Stammzelle in zwei identische Zellen teilt, bleibt eine unverändert zurück, während sich die andere Zelle zu mehr spezialisiereten Zelltypen entwickelt. Dies birgt großes therapeutisches Potenzial, da die Zellen Gewebe wiederherstellen und heilen können.

Blutbildende Stammzellen werden routinemäßig bei der Stammzelltransplantation eingesetzt, beispielsweise nach Chemotherapie bei Krebsbehandlungen. Ein weiterer Stammzelltyp, der im Knochenmark und in anderen Geweben vorkommt, birgt großes Potenzial für die regenerative Medizin, da die Zellen die Heilung unterstützen und sich in spezialisiertere Zelltypen wie Knochen, Knorpel oder Fett umwandeln können.

Blut ist ein weiterer wichtiger Bestandteil des Körpers und ermöglicht den Transport von Zellen, Nährstoffen, Sauerstoff und anderen molekularen Bestandteilen sowie Abfallprodukten. Es zirkuliert kontinuierlich durch unseren Körper und macht 7-8% des erwachsenen Körpergewichts aus. Blut besteht aus einer flüssigen Komponente, dem Plasma und verschiedenen Zelltypen: den roten Blutkörperchen, den weißen Blutkörperchen und den Blutplättchen, die alle verschiedene wichtige Funktionen haben. Rote Blutkörperchen transportieren Sauerstoff von der Lunge in den Körper und Kohlendioxid zurück in die Lunge. Weiße Blutkörperchen sind Teil des Immunsystems und schützen uns vor gesundheitsschädigenden Bakterien, Viren und

anderen schädlichen Substanzen. Blutplättchen sind wichtig dür die Wundheilung. Darüber hinaus enthält Blut viele wichtige Informationen über Körperfunktionen, da es mit allen Teilen des Körpers in Kontakt kommt und Immunzellen enthält, die erste wichtige Hinweise auf Erkrankungen geben können.

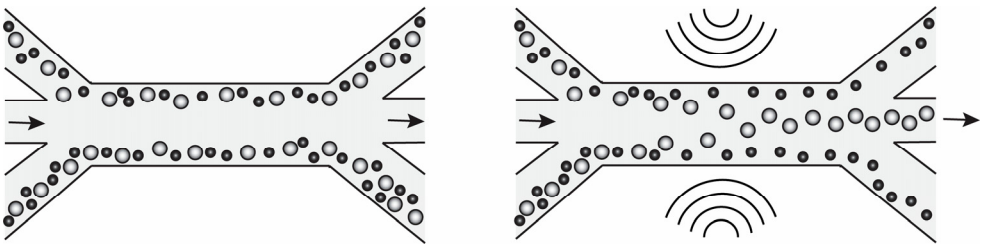

Abbildung 26: Vereinfachtes Princip der Akustophorese. Die linke Abbildung zeigt Zellen, die von links nach rechts durch einen Mikrokanal fließen, wie durch die Pfeile angezeigt. Größere (helle) und kleinere (dunkle) Zellen treten gemischt durch die Seitenkanäle ein, folgen dem Fluss und verlassen sie durch die Seiten an den gegenüberliegenden Ausgängen weiterhin gemischt wie beim Eintritt. Rechts wirkt ein akustisches stehendes Wellenfeld im Kanal und die größeren Zellen werden stärker von der akustischen Kraft beeinflusst. Deshalb bewegen sie sich in Richtung der Kanalmitte, während die kleineren Zellen weniger von den akustischen Kräften beeinflusst werden und nahe an den Kanalwänden bleiben. Die kleinen Zellen treten wie zuvor durch die Seitenzweige aus, während die größeren Zellen in der Mitte gesammelt werden. Dies resultiert in der Separation der beiden Zelltypen.

Die moderne Medizin hat einen langen Weg zurückgelegt. Wir wissen bereits viel darüber, wie der Körper funktioniert, was bei verschiedenen Krankheiten vor sich geht und wie sie behandelt werden können. Es gibt jedoch weiterhin viele Geheimnisse zu untersuchen.

Um verschiedene Blut- oder Knochenmarkkomponenten nutzen zu können und besser zu verstehen, ist es häufig erforderlich, bestimmte Zelltypen anzureichern oder andere aus diesen komplexen Materialien zu entfernen.

Diese Doktorarbeit untersucht die Verwendung einer Technologie namens Akustophorese zur Trennung von Blut- und Stammzellpräparaten. "Akustophorese" bedeutet im Grunde genommen Migration (Bewegung) durch Schall. Im Fall besagter Technologie bezieht sich dies auf Ultraschall, mit dem Zellen in Mikrokanälen manipuliert werden können. Wenn die Zellen unterschiedliche physikalische Eigenschaften aufweisen, wie zum Beispiel Größe, Dichte und Kompressibilität, bewegen sie sich entweder zu unterschiedlichen Positionen im Mikrokanal oder mit unterschiedlichen Geschwindigkeiten in einem stehenden Ultraschallwellenfeld (Abbildung 26). Dies ist auch abhängig von den Eigenschaften der umgebenden Flüssigkeit. Das Verhalten einer Flüssigkeit kann bei Verwendung eines Mikrokanals mit einer Breite von weniger als einem halben Millimeter genau vorhergesagt werden. Dann wird die Position der Zellen nur in Abhängigkeit von den Kräften im akustischen Feld bestimmt. Daher ermöglicht es, Zellen kontrollierbar zu manipulieren und

voneinander zu separieren, ausschließlich aufgrund von Unterschieden in ihren physikalischen Eigenschaften.

Zunächst wurde gezeigt, dass Akustophorese verwendet werden kann, um zirkulierende Tumorzellen aus Blutproben anzureichern und um sie aus Stammzelltransplantaten zu entfernen. Dies kann das Rückfallrisiko von Neuroblastom-Patienten verringern, indem lebende kontaminierende Tumorzellen nicht mit dem Zelltransplantat transplantiert werden. Das Neuroblastom ist in schweren Fällen, welche etwa 50% aller Patienten ausmachen, ein sehr tödlicher Krebs im Kindesalter. Angereicherte Tumorzellen könnten verwendet werden, um die Behandlung zu überwachen und den vorhandenen Tumor besser zu verstehen.

Darüber hinaus wurde untersucht, wie Akustophorese verwendet werden kann, um Stammzellen aus dem Knochenmark für verschiedene Anwendungen zu separieren. Da Stammzellen sehr selten sind, ist es notwendig, diese Zellen im Labor weiter zu züchten, um ausreichende Zellzahlen für die Transplantation zu generieren. Während des Expansionsprozesses können die Zellen einige ihrer Eigenschaften ändern oder verlieren, wobei manche Zellen ihre wichtigen Funktionen länger als andere beibehalten. Für die Transplantation ist es sehr wichtig, dass die Zellen gut charakterisiert und definiert sind. Hier wurde Akustophorese verwendet, um Unterschiede in kultivierten Stromazellen besser zu verstehen und somit Zellen mit verbesserten Eigenschaften zu identifizieren und zu isolieren.

Eine Herausforderung in der Stammzellforschung besteht darin, diese seltenen Zellen aus Gewebe zu isolieren. Hierin wurde gezeigt, dass Akustophorese verwendet werden kann, um diese seltenen primären Stromazellen aus frischen Knochenmarksproben anzureichern. Dies ist nützlich, um ein besser definiertes Ausgangsmaterial für die Zellexpansion im Labor zu erhalten. Darüber hinaus könnte Akustophorese als erster Anreicherungsschritt verwendet werden, um die Zeit zu verkürzen, die erforderlich ist, um diese seltenen Zellen für Forschungszwecke weiter zu isolieren, was ansonsten teure oder zeitaufwendige Methoden erfordert.

Um Akustophorese für die Stammzellprozessierung in Forschung und klinischen Anwendungen implementieren zu können, ist es besonders wichtig, den Zelldurchsatz zu erhöhen. Daher wurde untersucht, wie die Durchsatzrate der akustischen Zellseparation verbessert werden kann. Eine verkürzte Prozesszierungszeit wurde erfolgreich für die Separation verschiedener Arten weißer Blutkörperchen voneinander demonstriert.

Es kann arbeitsintensiv sein zu untersuchen, ob verschiedene Zelltypen anhand von Unterschieden in ihren unbekannten physikalischen Eigenschaften getrennt werden können. Daher wurde eine Methode entwickelt, um die akustischen Eigenschaften verschiedener Zelltypen basierend auf Separationsdaten und statistischen Analysen abzuschätzen. Dies ist sehr nützlich, um vorherzusagen, wie sich verschiedene Zelltypen in einem akustischen Feld verhalten werden. Daher kann bewertet werden, ob es möglich ist, Zelltypen voneinander zu separieren und was optimale experimentelle Bedingungen sein könnten. Dies ermöglicht es, Experimente mit einem optimierten

Akustophoresesystem zu beginnen, anstatt zeitaufwändige Trial-and-Error-Ansätze zu verfolgen.

Die Erkenntnisse dieser Doktorarbeit liefern daher einige neue Einblicke in die Anwendbarkeit von Akustophorese für die Prozessierung von Stammzellpräparaten. Das Verfahren ermöglicht es, Zellen basierend auf ihren akustischen Eigenschaften auf kontrollierbare, kontinuierliche und skalierbare Weise zu trennen. Die Technologie weist großes Potenzial auf, um möglicherweise in mehreren Forschungs- und klinischen Anwendungen implementiert zu werden.

Acknowledgements

"Nothing in life is to be feared, it is only to be understood. Now it is the time to understand more, so that we may fear less."
Marie Skłodowska-Curie

I think this quote, by one of the greatest female researchers of all times, is not only relevant for research, but also for life's and everyone's personal journey. It is hard to believe that it was almost 6 years ago that I emptied my apartment in Berlin, packed some things to finally open my mental wings to embark on my personal international Swedish adventure. Looking back, I would have never dared to imagine how life changing this experience would be.

I got to know great people, added new family members, and dear friends, learnt a lot about research, science, the world, and maybe most importantly about myself.

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