Usability and Applicability of Microfluidic Cell Culture Systems

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Usability and Applicability of Microfluidic Cell Culture Systems

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Abstract

Microfluidic cell culture has been a research area with great attention the last decade due to its potential to mimic the in vivo cellular environment more closely compared to what is possible by conventional cell culture methods. Many exciting and complex devices have been presented providing possibilities for, for example, precise control of the chemical environment, 3D cultures, controlled co-culture of different cell types or automated, individual control of up to 96 cell culture chambers in one integrated system. Despite the great new opportunities to perform novel experimental designs, these devices still lack general implementation into biological research laboratories.

In this project, the usability and applicability of microfluidic cell culture systems have been investigated. The tested systems display good properties regarding optics and compatibility with standard laboratory equipment and procedures. Such highly usable systems were achieved by integration of fluidic actuation, liquid reservoirs, liquid interconnections and cell culture chambers in a single portable unit. However, improvements regarding robustness of individual system parts and thereby reliability of the systems are shown to be needed. This is possibly one of the reasons for the lack of implementation of microfluidic cell culture systems into biological research laboratories.

Procedures to perform long-term microfluidic perfusion cell culture experiments have been established. Furthermore, successful application of the microfluidic perfusion cell culture system is shown by investigation of adipose-derived stem cell (ASC) differentiation into adipocytes, where we have revealed that paracrine/autocrine signaling is involved in differentiation of a population of ASCs into adipocytes. We have thereby demonstrated that microfluidic perfusion cell culture systems are a more powerful and a useful tool to investigate paracrine/autocrine signaling within a cell population compared to conventional static cell culture. Thus, we have been able to perform novel biological research by the use of our developed microfluidic perfusion cell culture systems.
Resumé

Mikrofluid celledyrkning har det sidste årti været et forskningsområde med stor opmærksomhed på grund af dets potentiale for nøjere at kunne efterligne de in vivo cellulære omgivelser i forhold til hvad, der er muligt ved konventionelle celledyrkningsmetoder. Mange spændende og komplekse devices, som giver mulighed for, for eksempel, præcis kontrol af de kemiske omgivelser, 3D kulturer, kontrolleret samdyrkning af forskellige typer celler eller automatiseret individuel kontrol af op til 96 celledyrkningskamre i ét integreret system, er blevet præsenteret. Til trods for de storartede nye muligheder for at udføre hidtil ukendte forsøgsopsætninger mangler disse devices stadig at blive generelt implementeret i biologiske forskningslaboratorier.


Procedurer for at udføre langtids-mikrofluide perfusions celledyrkningsexperimenter er blevet fastsat. Ydermere er en succesfuld anvendelse af mikrofluid perfusions celledyrkningsystemet vist ved undersøgelse af fedt-afledte stamcellers differentiering til fedtceller, hvor vi har afsløret at parakrin/autokrin signalering er involveret i differentiering af en fedstamcellepopulation til fedtceller. Vi har dermed demonstreret at mikrofluid perfusions celledyrkningssystemer er et mere kraftfuldt og brugbart redskab til at undersøge parakrin/autokrin signalering indenfor en cellepopulation i forhold til konventionel statisk celledyrkning. Vi har således været i stand til at udføre ny biologisk forskning ved brug af vores udviklede mikrofluid perfusions celledyrkningssystemer.
Preface

This thesis is submitted in partial fulfillment of the requirements for obtaining the Ph.D. degree at the Technical University of Denmark. The work has been carried out in the Fluidic Array Systems and Technology (FAST) group at the Department of Micro- and Nanotechnology (DTU Nanotech), Technical University of Denmark (DTU) in the period from March 2009 to March 2012. The work has been supervised by main supervisor assoc. prof. Martin Dufva and co-supervisor prof. Philippe Collas, University of Oslo. The project has been financed and carried out within the framework of the NABIIT project ProCell.

Many people have helped, guided, inspired and motivated me through this project. First of all, I would like to thank my main supervisor Martin Dufva for always being helpful with useful and inspiring discussions of results and further directions. Furthermore, I have really appreciated your frequent visits in the lab to look at cells and running or not running devices during the experimental work. Regarding the stem cell work, I would also like to thank Philippe Collas and Anita Sørensen, University of Oslo, for providing the stem cells and for discussions and evaluation of the stem cell differentiation results obtained in this project.

My fellow Ph.D. student within the ProCell project, Peder Skaft-Pedersen, has been of invaluable help by introducing me to many aspects in microfluidics, microfluidic device fabrication, microscopy and image processing/analysis. Furthermore, I would like to thank Peder for a really nice company during the lab work. Also Joanna Lopazinska, David Sabourin and Maciej Skolimowski have been very helpful within microfluidic cell culture and system component fabrication. Other thanks are given to Søren Vedel and Henrik Bruus for introducing me to theoretical microfluidic concepts and comments on flow and mass transport issues in my Ph.D. thesis. Finally, I want to thank my officemates Maria, Joanna, Alvaro and Maciej and all other people and students at DTU Nanotech for a really nice and inspiring working atmosphere.

At last but not least, I want to thank my family and friends for supporting me during this Ph.D. study. Special thanks are given to my husband and two daughters for given me the opportunity to go through a Ph.D study; I know I have been very absent the last year. I also want to give a special thanks to my parents for always supporting me when I needed it.

Mette Hemmingsen
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<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>Adipogenic medium</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose-derived stem cell</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic proteins</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT-enhancer-binding protein alpha</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CPR</td>
<td>Critical perfusion rate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>Dkk1</td>
<td>Dickkopf-1</td>
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<tr>
<td>DS</td>
<td>Densitometric sum</td>
</tr>
<tr>
<td>DTU</td>
<td>Technical University of Denmark</td>
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<tr>
<td>DTU Nanotech</td>
<td>Department of Micro- and Nanotechnology</td>
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<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>ECT</td>
<td>Effective culture time</td>
</tr>
<tr>
<td>ECV</td>
<td>Effective culture volume</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
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<tr>
<td>FABP4</td>
<td>Fatty acid binding protein 4</td>
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<tr>
<td>FAST</td>
<td>Fluidic Array Systems and Technology</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FZD1</td>
<td>Frizzled1</td>
</tr>
<tr>
<td>FZD2</td>
<td>Frizzled2</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
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<tr>
<td>GM</td>
<td>Growth medium</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobuthyl-methylxanthine</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
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<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>KLF</td>
<td>Kruppel-like factor</td>
</tr>
<tr>
<td>LAF</td>
<td>Laminar air flow</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>LRP5/6</td>
<td>Low-density lipoprotein receptor-related protein-5 or -6</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
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<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>mESC</td>
<td>Mouse embryonic stem cells</td>
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<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
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<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<td>NCS</td>
<td>Newborn calf serum</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>Polycarbonate</td>
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<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
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<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methylmethacrylate)</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PTFE</td>
<td>Poly (tetrafluoroethylene)</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SAV</td>
<td>Surface area to volume</td>
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<td>SCREBP1c</td>
<td>Sterol response element-binding protein 1-c</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error on the mean</td>
</tr>
<tr>
<td>sFRP</td>
<td>Secreted frizzled-related proteins</td>
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<td>SHN2</td>
<td>Schnurri 2</td>
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<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TCF7L1</td>
<td>Transcription factor 7-like1</td>
</tr>
<tr>
<td>VBA</td>
<td>Visual Basic for Applications</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-type mouse mammary tumor virus (MMTV) integration site family members</td>
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<tr>
<td>ZFP</td>
<td>Zinc-finger protein 423</td>
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Chapter 1

Introduction

In vitro cell culture is a central method within biological/biomedical research that has formed the basis for most of the knowledge obtained about cell biology during the last century. For example, in vitro cell culture has been of great value in unraveling signaling pathways, studying cytoskeletal arrangements/rearrangements, and drug screening. In general, cell culture is performed by the use of petri dishes, flasks or well plates of different sizes, in which adherent cells are cultured in a 2D-monolayer covered with roughly 1.5 to 2 mm of cell culture medium. Except for optimizations of cell culture medium compositions and improvements of the substrates, the method of in vitro cell culture has not changed significantly since its introduction.

One advantage of the existing method is that it keeps the experimental conditions simple and fairly well-controlled with respect to parameters that can affect the cellular response. Often a stimulating or inhibitory factor is added to the cell culture medium, and the cellular response is assayed on average conditions. In addition, replicates can be obtained for statistics using a reasonable amount of time and effort. However, the present method of in vitro cell culture mimics the in vivo cellular environment poorly. In vivo, the cells are in a complex 3D environment in which they are influenced by many different soluble factors, cell-extra cellular matrix (ECM) interactions, and direct cell-to-cell contacts. Interpretation of the results using the existing cell culture method and transfer of the results to what is happening in in vivo situations must therefore be made with caution.
However, during the last decade a new innovative approach to in vitro cell culture has emerged in the form of microfluidic cell culture.

1.1 Microfluidic cell culture

Microfluidic technology is the ability to control and manipulate very small volumes of liquids in channels and chambers in the micro scale [1]. The technology has been widely applied to mammalian cell culture [2], leading to the term microfluidic cell culture. The geometries in the micrometer scale and volumes in microlitres by themselves mimic the in vivo cellular environment more closely than conventional cell culture methods and thereby probably increase the biological relevance. Furthermore, one very important advantage is the ability to control the chemical composition of the near-cell environment, also called the cellular microenvironment. This precise control has made it possible to stimulate cells at the sub cellular level [3] and generate stable continuous gradients of soluble factors for cell exposure [4].

The research in microfluidic cell culture can be divided into a few main focus areas. In addition to high accuracy flow control systems as mentioned above, many scientific groups strive to develop high-throughput cell culture systems, which are either very complex or simpler in design. Quake et al. [5] presented an integrated automated system with mixers, peristaltic pump, pneumatic valves, and 96 cell culture chambers that could be controlled individually. A simpler approach to high-throughput was demonstrated by Beebe et al. [6] in the form of a tubeless array of 192 cell culture chambers in standard micro titer plate format and with flow control by passive pumping.

In an attempt to mimic the in vivo cellular microenvironment further, many studies aim to culture and analyze cells in 3D ECM-like environments. A device presented by Toh et al. [7] enabled cell-cell interactions through cell seeding in an array of micropillars capturing the cells. Furthermore, cell-ECM-like interactions were obtained by the formation of 3D collagen matrices surrounding the cells. Another study [8] investigated proliferation and differentiation of human mesenchymal stem cells embedded in 3D poly(ethylene terephthalate)(PET) scaffolds under static and perfusion culture conditions. 3D cell culture and high-throughput experimentation were combined on a microfluidic passive perfusion array in a 96-well format consisting of 32 independent flow chambers, presented by Chen et al. [9].

Similar to 3D culture systems, co-culture devices have been presented that allow the study of one cell type behavior in relation to another cell type. Intravascular adhesion of flowing metastatic breast cancer cells to chemokine activated endothelium has been investigated by using a membrane
integrated device [10]. Endothelial cells were cultured on the membrane and activated from the basolateral side through the membrane by chemokines, and attachment of the floating cancer cells to the apical side was assayed.

Most recently, the trend in microfluidic cell culture research has moved towards tissue engineering, with the aim of mimicking in vivo conditions even further. For example, by co-culturing stromal cells and endothelial cells under physiological interstitial flow conditions in a microfluidic device, a vascular network with perfused capillaries was shown to be created within 18 days of culture [11]. Another recent study demonstrated the formation of proximal-distal patterning of mouse epiblast by spatially micro patterning two different cell types, thereby creating two different extraembryonic environments [12].

Overall, the ultimate goal of the microfluidic cell culture research community has been and still is to develop devices/systems that mimic the in vivo cellular microenvironment as much as possible and at the same time enable controllable dynamic cell stimulation and high-throughput cell analysis.

1.2 Motivation

As described in section 1.1, the development of microfluidic cell culture has been rapid and has already provided many new possibilities for in vitro cell culture, by mimicking in vivo conditions more closely and/or by improving the control of the cellular microenvironment. Despite these great new opportunities for bringing more biological relevance into biological/biomedical research, the methods have not been adopted by biologists and biological/biomedical labs in general. In my opinion, most biologists do not even know about the possibilities that are offered by the microfluidic research community. This lack of implementation is well known within the microfluidic research community and has been discussed in a few reviews/letters [1, 13, 14]. It is therefore of crucial importance that systems/devices are developed in close collaboration with the biologists and finally evaluated by the biologists. In addition, papers presenting new devices and applications, in general, only describe the successful experiments, but lack the reporting of failure rates and time consumptions. Although not described, this is however of great significance for the end users and the implementation into biomedical research. For example, in a presentation of a microfluidic cell culture device, it can be emphasized that the device is applicable to long-term cell culture experiments (the cell culture has been running for up to two weeks). But how many cell culture experiments have been tried to be run for two weeks, and how many of those failed before the two weeks. If the successful experiments are only three out of ten, for example, then the device is almost useless. Such a
high failure rate can normally not be accepted by a biologist. Thus, in this project, we want to investigate the usability and the applicability of microfluidic cell culture within biological/biomedical research.

**1.3 Project objectives**

This project had two main objectives:

1. **Evaluation of microfluidic cell culture systems, system components, and handling procedures seen from an end user’s point of view.** Based on obtained working experiences, design considerations and handling procedures for microfluidic cell culture systems are to be discussed.

2. **Application of microfluidic cell culture to investigate a biological issue.** Most previously presented work is proof-of-concepts. However, the aim of this project is to move a step further and employ microfluidic cell culture as a method/tool in biological/biomedical research.

**1.4 Project basis**

**1.4.1 The ProCell project**

This project is a part of the ProCell project (Grant No. 2106-08-0018 "ProCell", under the Programme Commission on Strategic Growth Technologies, the Danish Agency for Science, Technology and Innovation) and has been performed within the framework of the ProCell project. The ProCell project is a highly interdisciplinary collaboration between biology, micro/nanotechnology and information technology, with its main partners from the Department of Micro- and Nanotechnology and the Department of Informatics and Mathematical Modeling, both from Technical University of Denmark. ProCell is an abbreviation of ‘Programmable cell culture chip’ and the goal of the project has been to design and fabricate a device for parallel and combinatorial cell culture experiments with the capability of real-time monitoring and manipulation of the cells (Fig. 1). The experimental design and performance were aimed to be aided by the development of computer-assisted user-programmable control programs and theoretical models for the physical and chemical properties of the microfluidic environment.

In relation to this PhD project in particular, one of the ProCell objectives regarding evaluation was to test the developed device for investigation and optimization of the differentiation of stem
cells in collaboration with the external academic partner, Professor Philippe Collas, University of Oslo. The design and fabrication of the microfluidic cell culture device has mainly been performed by Peder Skafte-Pedersen, and this work is presented in his PhD thesis entitled “Microfluidic Cell Culture Systems for Real-time Studies of Cells” [15]. The system developmental work has been carried out in parallel with the biological testing/application of the system and in close collaboration and sparring with the author of this thesis. For example, the cell loading has been improved by a suggestion to reduce the pathway from the cell loading well to the cell culture chamber.

![Image](www.nanotech.dtu.dk/ProCell)

**Fig. 1.** Overview of the proposed ProCell platform combining biology, micro- and nanotechnology and information technology. From www.nanotech.dtu.dk/ProCell.

### 1.4.2 The FAST research group

This project has been carried out within the Fluidic Array Systems and Technology (FAST) group, headed by Associate Professor Martin Dufva. During the last decade, the research focus area of the FAST group has moved towards microfluidic cell culture and analysis, initiated with the development and characterization of a biocompatible single chamber cell culture chip by Michael Stangegaard [16-18]. Fluidic interconnections have been improved by the development of ball joint interconnection features by David Sabourin [19, 20] and integrated fluidic actuation has been obtained by the design and fabrication of a peristaltic micropump, carried out by Peder Skafte-Pedersen and David Sabourin [21]. Additional engineering has resulted in a whole library of components for the creation of various modular microfluidic systems tailored to specific applications ([Appendix 4](#)) (manuscript in preparation). The ProCell project has been based on the initial developments and has been a continuation of this research line.
1.5 Approaches and methodology

1.5.1 Evaluation of microfluidic cell culture systems

In general, the evaluation is based on working experiences obtained by the practical use of microfluidic cell culture systems and components designed and fabricated within the ProCell project and the FAST group.

This part of the thesis is divided into design considerations/performance of microfluidic cell culture systems as well as system components and evaluation of normal cell culture procedures. The evaluation of microfluidic cell culture systems and components has focused on general usability such as assembling, portability, robustness, compatibility with standard laboratory equipment and sterilization procedures, and reliability. Assessment of normal cell handling procedures in a microfluidic perfusion cell culture system has concentrated on sterilization and coating procedures, cell seeding, cell attachment and proliferation, transfection of DNA, as well as cell manipulation in terms of regulation of gene expression. Again, attention has been paid to general usability.

Overall, the evaluation has not focused on biocompatibility issues due to the fact that these have been intensively investigated previously by other members of the FAST group. Michael Stangegaard has compared cell proliferation and whole genome expression profile in a poly(methylmethacrylate) (PMMA) cell culture chamber to conventional cell culture methods [17, 18]. Biocompatibility of PMMA and poly(dimethylsiloxane) (PDMS) has been assessed with respect to cell viability and proliferation rate, cell morphology, cell cycle states, and DNA microarray based transcription profiling by Joanna Lopacinska and is presented in her PhD thesis entitled “Micro- and Nanoengineering Meets Cell Biology: In Vitro Biocompatibility Testing Concepts” [23].

The evaluation part of the work presented in this thesis distinguishes itself from previously presented work in the literature by being more critical, assessed from an end user’s point of view, and by reporting failure rates/time consumptions. In striving towards showing good performance and thereby getting papers accepted for publication, only the successful experiments are presented. However, with the poor adoption of microfluidic cell culture by biological/biomedical research labs in mind, one could speculate that this could be related to issues such as ease of use, robustness, and reliability. By reporting and focusing on these issues, the hope is to contribute to enhancing the implementation of microfluidic cell culture methods in biological/biomedical research.
1.5.2 Application of microfluidic cell culture to stem cell research

Stem cells have a promising potential for the generation of tissue models for basic biological research, biomedical research (as for example drug testing), and for regenerative cell therapies [24, 25]. The fate of the stem cell is controlled by many external cues, such as soluble factors, cell-ECM and cell-cell interactions, all acting in a complex network [26], which is difficult to copy by conventional cell culture methods. In comparison, microfluidic cell culture offers the potential to more closely mimic the in vivo conditions and has therefore been widely applied to stem cell research [27, 28]. However, most studies have been proof-of-concepts [29-33], although more recent work has moved a step further and tried to investigate issues within the stem cell area [34-36]. In this project, we wanted to continue this progress of the application of microfluidic cell culture within stem cell research.

Accordingly, one of the objectives of the ProCell project and thereby this project was, in collaboration with the external academic project partner, Professor Philippe Collas, to apply the developed microfluidic cell culture systems to investigate and optimize the differentiation process of adipose-derived stem cells (ASCs) into adipocytes (mature fat cells). The established protocol [37] for differentiation of ASCs into adipocytes dictates that the cells should be cultured to 90 % confluence and then medium changed to adipogenic medium consisting of 4 different inducers of differentiation. The differentiation process continues for up to 3 weeks with medium change twice a week. The fact that the cells must be almost confluent at the time of induction of differentiation indicates involvement of cell-to-cell signaling, either by direct cell-to-cell contact or by paracrine/autocrine signaling. Depending on the flow rate, perfusion cell culture may disrupt paracrine/autocrine signaling by constantly removing cell released factors from the near-cell environment. In this project, we wanted to investigate the effect of different flow rates and chemical compositions of the adipogenic medium on the differentiation process at various cell densities.

1.6 Main findings

1.6.1 Usability of microfluidic cell culture systems

Regarding usability, the tested microfluidic cell culture systems displayed good properties regarding optics and compatibility with standard laboratory equipment and cell handling procedures. The high usability of the systems was achieved by integration of fluidic actuation, liquid reservoirs, liquid interconnections and cell culture chambers in a single portable unit. However, improvements
Chapter 1

regarding robustness of individual parts and thereby reliability of the systems is shown to be needed.

1.6.2 Applicability of microfluidic cell culture systems

The tested microfluidic perfusion cell culture systems have successfully been applied to investigate adipose-derived stem cell (ASC) differentiation into adipocytes. By microfluidic perfusion cell culture, we have revealed that paracrine/autocrine signaling is involved in differentiation of a population of ASCs into adipocytes. Furthermore, we have demonstrated that microfluidic perfusion cell culture systems are a more powerful and a useful tool to investigate paracrine/autocrine signaling within a cell population compared to conventional static cell culture.

1.7 Thesis outline

The content of this thesis is selected work performed during the three-year PhD study, presented together with an introduction to the different areas and a discussion of the results. The most important results are, in addition, presented in the first-author or co-author papers attached as appendices to the thesis. Below are listed the chapter headings, a short description of the content of each chapter and a statement of the work presented in first-author and co-author papers:

Chapter 2: Background/Theory

Basic concepts and issues related to microfluidic cell culture are presented, as well as the cellular environment in vitro contra in vivo and cell-to-cell signaling aspects. In addition, the basic knowledge about the differentiation process of stem cells into adipocytes is described.

Chapter 3: Materials and Methods

Used methods, protocols, and materials are presented in a condensed form.

Chapter 4: System Design Considerations and Reliability

Different approaches to design and fabrication of microfluidic cell culture devices are shortly described, followed by an investigation and evaluation of systems and system components developed in the ProCell project and the FAST group. The aspects considered are general usability such as assembling, portability, robustness, compatibility with standard laboratory equipment and procedures, and reliability. The outcome of the microfluidic cell culture system investigation and evaluation is included in co-author manuscript no. 2.
Chapter 5: Microfluidic Perfusion Cell Culture

Different approaches to normal cell handling procedures related to microfluidic perfusion cell culture are shortly presented followed by procedures employed and results obtained during this project. The procedures include sterilization and coating procedures, cell seeding and perfusion cell culture. Furthermore procedures for transfection of DNA and regulated gene expression of a fluorescent reporter protein with real-time detection are presented. The optimization work presented in this chapter has been conducted in parallel and in interaction with the system development and forms the basis for the system application presented in co-author paper no. 1.

Chapter 6: Application to Stem Cell Research

Application of microfluidic cell culture to stem cells is briefly presented. This is followed by results achieved during this project by investigation of ASC differentiation into adipocytes in perfusion cell culture at different flow rates, adipogenic medium compositions and various cell densities. This work has been presented in conference proceeding no. 1 and is going to be further presented in a first-author manuscript together with theoretical modeling of fat accumulation in relation to applied flow rates, diffusible signaling, and supply of nutrients provided by the theoretical partners in the ProCell project.

Chapter 7: Overall Conclusion and Outlook

A conclusion of achieved results is presented followed by speculations/suggestions for future applications of microfluidic cell culture.

1.8 Publications

Below are presented peer reviewed papers and conference proceedings either as first-author or co-author work. The status of the publications is given as published, submitted, prepared for submission or in preparation.

1.8.1 Manuscripts and papers for peer reviewed journals

1. Peder Skafte-Pedersen, Mette Hemmingsen, David Sabourin, Felician Stefan Blaga, Henrik Bruus, and Martin Dufva: A self-contained, programmable microfluidic cell culture system with real time microscopy access. Published online in Biomedical Microdevices, 13 December 2011.
Chapter 1

2. David Sabourin, Peder Skaft-Pedersen, Vasile Coman, Mette Hemmingsen, Jesper Petersen, Jenny Emneus, Jörg P. Kutter, Detlef Snakenborg and Martin Dufva: **MainSTREAM: a modular and scalable microfluidic platform.** In preparation.

1.8.2 Peer reviewed conference proceedings

1. Mette Hemmingsen, Peder Skaft-Pedersen, David Sabourin, Rasmus Find Andersen, Anita L. Sørensen, Philippe Collas, and Martin Dufva: **Perfusion cell culture reveals a paracrine or autocrine signaling pathway involved in adipose-derived stem cell differentiation into adipocytes.** Published in Proceedings of the Fifteenth International Conference on Miniaturized Systems for Chemistry and Life Sciences, pp. 834-836, 2011.

2. Wajid Hassan Minhass, Paul Pop, Jan Madsen, Mette Hemmingsen, Peder Skaft-Pedersen, Martin Dufva: **Cell Culture Microfluidic Biochips: Experimental Throughput Maximization,** 5th International Conference on Bioinformatics and Biomedical Engineering, (iCBBE), 2011.


Chapter 2

Background/Theory

This thesis spans microfluidic cell culture systems and stem cell differentiation. It may therefore be read by people with a background in physics/engineering or biology. To assist the reading and interpretation of the results some background knowledge is presented in this chapter. This includes basic knowledge about the cellular microenvironment in vivo contra in vitro. Furthermore, concepts/issues related to microfluidic cell culture are presented, including aspects of soluble factor signaling between cells. Finally, some basic knowledge about stem cells and, in particular, the established knowledge about the differentiation process of stem cells into adipocytes is presented.

2.1 The cellular microenvironment

The human cellular microenvironment and the cellular response hereof are of course best studied within its natural environment, i.e. the human body. However, due to practical, safety and ethical issues, this is normally not possible, and instead other biological models have been widely employed. The most common are experimental animals, tissue slices from the animals, and in addition, cells isolated from different tissues, such as skin, brain, liver, lung, breast, the intestine or the vascular system. The isolated cells are cultured in vitro in a dish/flask with optimized medium composition and maintained inside an incubator with proper conditions regarding temperature, CO₂ and humidity. Although simple, and therefore widely used, in vitro cell culture differs significantly from in vivo conditions with respect to many parameters that must be taken into account when interpreting the data. This inadequacy of the current in vitro cell culture methods has been the main motivator for the development of more in vivo-like methods within the microfluidic research com-
munity. Below in section 2.1.1 and 2.1.2 are described some important differences between the \textit{in vivo} and \textit{in vitro} cellular microenvironments.

\textbf{2.1.1 The \textit{in vivo} cellular microenvironment}

\textit{In vivo} the cells are in a three dimensional configuration adhered to the ECM, with short distances from one cell to a neighbour cell and with the extra-cellular volume less than the cell volume \cite{26, 38-40} (\textit{Fig. 2a} and \textit{b}). Nutrients are constantly supplied to the cells and waste removed from the near-cell environment. Furthermore, the cells are continuously affected by cues in the microenvironment, the concentration of which can vary over time and space. The parameters that affect the cells can be biochemical, physical or physiochemical in character.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{The \textit{in vivo} cellular microenvironment. \textit{a)} Simplified drawing of the \textit{in vivo} cellular environment depicting various ways of cell-to-cell signaling. \textit{b)} Liver tissue slice showing the tight cell conformation and low ratio of external fluid volume to cell volume. Image from www.leica.microsystems.com.}
\end{figure}

The biochemical signaling can take place via cell-ECM interactions by for example integrins that connect the cell to the ECM and transfer signals from the ECM to the interior of the cell. Another type of cell-to-cell signaling happens directly through cell-to-cell junctions, and finally a major part of the cell-to-cell signaling is carried out by paracrine or autocrine signaling. The cells secrete soluble factors such as cytokines, growth factors and hormones that are sensed by the same cell (or same cell type, called autocrine signaling) or a neighbour cell (often another cell type, called paracrine signaling) (\textit{Fig. 2a}). The signal to the target cell is sensed by receptor binding, endocytosis or diffusion through the cell membrane determined by the nature of the signaling molecule. This soluble factor signaling is dependent on diffusion, stability and binding affinity to a possible receptor of the signaling molecule.

The physical effects can be in the form of shear stress, different topographies and substrate stiffnesses, which have been shown to influence, for example, the fate of stem cells \cite{39, 41}. The pH, oxygen tension, temperature and osmolarity surrounding the cell define the physiochemical
environment. For example hypoxia in the primary tumour has been observed to be associated with cancer metastasis [42].

2.1.2 The in vitro cellular environment in conventional cell culture methods

In the conventional method of in vitro cell culture, the cells are cultured in a two dimensional monolayer in a petri-dish or flask, normally made of polystyrene. For most of the culture period the cells have no direct cell-to-cell contact, and the cell culture is mostly a mono-culture, i.e. the culture of one cell type. Instead of a constant supply of nutrients, the cell culture medium is renewed periodically (normally every second or third day), and the volume of medium is very high compared to the cell volume. The high culture volume results in a higher dilution of secreted signaling molecules compared to the in vivo conditions, which may affect the cellular response in a different way. For example, for some signaling a certain threshold may be necessary to induce the cellular response. The procedures including a high cell culture volume, incubation and periodic change of medium or cell passaging mean that the cell culture conditions, in general, change over time.

2.2 Microfluidic cell culture aspects

As mentioned in the introduction to section 2.1, the inadequacy of the current macro-scale in vitro cell culture methods to mimic the in vivo conditions has been the main motivator for the development of more in vivo-like methods within the microfluidic research community. The many possibilities and potentials offered by microfluidic cell culture, such as control of the chemical environment, 3D cultures and co-cultures (described in section 1.1), mimic more closely the in vivo cellular microenvironment. However, many phenomena and aspects are different from macro scale cell culture to microfluidic cell culture. Thus, it is important to be aware of the nature of those differences to be able to interpret and evaluate biological data obtained by the use of microfluidic cell culture devices.

2.2.1 Flow

In microfluidic cell culture systems, the cells are often constantly perfused with cell culture medium. The constant perfusion of the cell culture chambers ensures that nutrients are continuously supplied and waste products removed (Fig. 3). However, constant perfusion of the cell culture chamber means that signaling molecules, secreted by the cells, are probably washed away, which thereby interrupts a possible autocrine/paracrine signaling (Fig. 3b). This issue is further commented on in section 2.2.2 and chapter 6.
Whether the flow is laminar or turbulent is described by the Reynolds number, which is defined by [43, 44]

\[ Re = \frac{\rho v D_h}{\mu}, \]  

where \( \rho \) is the fluid density, \( v \) is the characteristic velocity of the fluid, \( \mu \) is the fluid viscosity, and \( D_h \) is the hydraulic diameter. The hydraulic diameter is a computed value that is dependent on the cross-sectional dimensions of the channel. In general, a Reynolds number lower than 2300 [43, 44] indicates that the flow is laminar. All the microfluidic cell culture experiments carried out in this project have been performed at constant perfusion conditions. The calculated Reynolds number for the employed chamber dimensions and flow conditions is in the range from \( 10^{-3} \) to \( 10^{-1} \) [15], and the flow is therefore dominated by laminar flow.

**Fig. 3. Simplified sketch of the flow effects on the chemical microenvironment.** a) At low flow some small cell secreted signaling molecules may be able to diffuse against the flow direction. However, at low flow the medium may be depleted for nutrients before new medium supply. b) At high flow the cells are supplied with new medium before depletion of nutrients. However, cell secreted signaling molecules will be washed away and thereby interrupt a possible paracrine/autocrine cell signaling.

### 2.2.2 Diffusion

Mass transport by diffusion is the dominant mass transport mechanism at long time scales and short distances [43]. Diffusion can be characterized by

\[ d^2 = q_i D t, \]  

where \( d \) is the distance that the particle diffuses in time \( t \) and \( D \) is the diffusion coefficient of the particle. \( q_i \) is a numerical constant which depends on the dimensionality. \( q_i = 2, 4, \) or 6 for 1, 2, or 3-dimensional diffusion. From equation (2) the diffusion time \( t \) can be written as

\[ t = \frac{d^2}{q_i D}. \]
As seen from equation (3), the time it takes a particle to move a certain distance is dependent on the squared power of the distance. The diffusion coefficient of a molecule can be estimated by the Stokes-Einstein relation

\[ D = \frac{k_B T}{6\pi \eta a}, \]  

(4)

where \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( \eta \) is the medium viscosity and \( a \) is the hydrated radius of the molecule. The diffusion coefficient \( D \) of a protein can be estimated by [45]

\[ D = 8.34 \times 10^{-12} \left( \frac{T}{\eta M^2} \right), \]  

(5)

where \( M \) is the molecular mass of the molecule and \( \eta \) is the medium viscosity. Because of the small dimensions within microfluidic cell culture, diffusion is more important than for macro scale cell culture, where diffusion is an extremely slow process. For example, for hemoglobin, with a diffusion constant in water of \( 7 \times 10^{-11} \) m\(^2\)/s, it takes \( 10^6 \) sec to diffuse 1 cm, but only 1 sec to diffuse 10 \( \mu \)m [44]. Whether the dominant mass transport mechanism is diffusion or convection can be determined by the Péclet number defined by [43]

\[ Pe = \frac{vl}{D}, \]  

(6)

where \( v \) is the velocity, \( l \) is the characteristic length and \( D \) is the diffusion coefficient of the particle. Mass transport by convection dominates at large Péclet numbers, while diffusion dominates at low Péclet numbers. Thus, the mass transport in a microchamber with laminar flow conditions is dominated by convective mass transport along the length axis of the chamber and by diffusion along the cross-sectional axis (Fig. 4a). As mentioned in section 2.2.1, the constant perfusion of the cell culture chamber will probably wash away signaling molecules secreted by the cells and thereby interrupt an autocrine/paracrine signaling. However, the removal of signaling molecules will depend on the applied flow rate. At low flow rates small molecules are able to diffuse against the flow direction and thereby stay within the near-cell environment (Fig. 3a). The diffusion coefficient of a molecule is inversely related to the radius of the molecule (equation 4). Thus, the degree of interruption of the paracrine/autocrine signaling is dependent on the flow rate and the size of the signaling molecule.
2.2.3 Surface area to volume ratio

The surface area to volume (SAV) ratio in microscale cell culture is much higher compared to macroscale cell culture. The SAV ratio of the cell culture microchamber dimensions used in this project is about 8.5 times larger than in a 6 well plate or a T75 flask (listed in Table 2). One drawback of the high SAV ratio in microchambers can be protein absorption or protein denaturation. A hydrophobic interior of a protein may be attracted to a hydrophobic surface of the chamber and thereby denature [43]. The large SAV ratio can also have an influence on the material/liquid interfaces. For that reason hydrophobic surfaces are often coated. In addition, diffusion of hydrophobic molecules into PDMS has been observed [46], which can be problematic due to the fact that many signaling molecules and drugs are hydrophobic. However, in a perfusion cell culture system quite large volumes of media are perfused through the system, thereby decreasing the total actual SAV. Furthermore, it can probably be assumed that the surfaces of the microfluidic network is not an endless sink of absorption of molecules, implying that a possible problem about a high SAV is probably non-existing in microfluidic perfusion cultures.

2.2.4 The effective culture volume

The concept ‘the effective culture volume’ (ECV) was introduced by Walker et al. [43] and describes the cells ability to control its environment. It is dependent on the mass transport along the x, y and z axes, whether the mass transport is dominated by diffusion or convection, the cell volume to culture volume (volume cell density) and possible protein absorption to the surface caused by a large SAV.

![Fig. 4: Simplified drawing of the effective culture volume in microscale culture and macroscale culture.](image)

*a) In microscale culture the mass transport is mainly by convection in the flow direction x and less by diffusion in the y and z direction. b) In macroscale cell culture the mass transport is in all three directions by both diffusion and convection. Adapted from [43].*
In microscale cell culture the ECV is smaller than in macroscale cell culture (Fig. 4). In the microcell culture chambers, at a seeding cell density of 100 cells/mm$^2$, the volume cell density is 4 times higher compared to macroscale (Table 1), and the mass transport is mainly by convection in the flow direction x and less by diffusion in the directions y and z (Fig. 4a). In macroscale cell culture the mass transport is in all three directions by both diffusion and convection, and the ratio of cell volume to culture medium volume is low (Table 1 and Fig. 4b). The low ECV in microscale culture mimics the in vivo conditions more closely than the conventional macroscale cell culture methods.

2.2.5 Perfusion flow rate

To ensure good cell viability and satisfactory cell proliferation, the cells need regular supply of nutrients and removal of cellular waste products. The most important components in the cell culture medium are growth factors and molecules needed for the cell metabolic processes, such as glucose. In static conventional cell culturing this is normally ensured by changing the cell culture medium every 2-4 days. In microfluidic perfusion culture, the question is how to determine the optimal perfusion flow rate. The concept ‘the effective culture time’ (ECT) was introduced by Young et al. [1] and describes the time between changes of the cell culture medium in the cell culture chamber. ECT is dependent on the initial concentration of a needed molecule, the reaction time of the molecule with the cells, the diffusivity of the molecule, the height of the cell culture chamber, the cell density, the cell culture area and the medium volume [1]. The diffusion time scale and the reaction time scale of a given molecule with the cell can be related by the Damkohler number $Da$ given by [1]

$$\frac{Da}{\tau_{Reac}} = \frac{\tau_{Diff}}{\tau_{Reac}} = \left(\frac{h^2}{D}\right)\left(\frac{C_0}{K_m\sigma}\right),$$

(7)

where $h$ is the height of the microchamber, $D$ the diffusion coefficient of the molecule, $C_0$ the initial concentration of the molecule, $K_m$ the maximum reaction/uptake rate of the molecule with the cell and $\sigma$ the cell density. In a diffusion dominant system, Young et al. [1] assume that the consumption of nutrients and growth factors is limited by the time scale of the reaction kinetics ($\tau_r$) with the cell rather than the diffusion time scales. Since they assume that $\tau_r$ is equivalent to ECT and $\tau_r$ thereby is linearly proportional to the height of the cell culture chamber ($h$), they suggest that ECT can be scaled according to $h$. Thus, if ECT in a macroscale chamber with a medium height of 1.5 mm is 48 hours, then ECT in a microchamber of height 0.5 mm is 16 hours.
To determine the optimal flow velocity in a perfusion system Young et al. [1] further introduced a dimensionless parameter $\kappa$ derived from the ratio of the perfusion time scale and the reaction time scale:

$$\kappa = \left( \frac{L}{U_m} \right) \left( \frac{C_{gh}}{K_{m\sigma}} \right) = \frac{L K_{m\sigma}}{U_m C_{gh}},$$

where $L$ is the length of the microchamber and $U_m$ is the flow velocity of the molecule. The lowest possible flow rate (named the critical perfusion rate (CPR)) to ensure replenishment of nutrients and growth factors before complete depletion could be estimated for $\kappa = 1$ and is then given by

$$\text{CPR} = \frac{L}{\tau_r} = \frac{L}{ECT},$$

where $L$ is the length of the chamber and ECT is the time between changes of medium scaled according to the height of the microchamber. However, the general assumption that $\tau_r$ is equivalent to ECT is a very rough assumption. The time between changes of medium in conventional cell culturing is based on recommendations from the supplier or personal experiences/opinions, but normally not based on experimental results. Thus, an experimental determination of the optimal flow velocity in a perfusion system based on cell viability and proliferation may be a better method compared to the theoretical computation presented by Young et al. [1].

### 2.2.6 Total culture volume

One of the often mentioned advantages of microfluidic cell culture is reduced costs for reagents because of lower cell culture volume. This is however very dependent on the applied/necessary flow rate. In a conventional static culture of ASCs, the medium is normally changed every four days. The medium consumption for, for example, a 6 well and 48 well plate is then 2 mL and 0.2 mL, respectively, while a perfusion cell culture of 500 nL/min or 125 nL/min consumes 2.8 mL or 0.72 mL per chamber after four days of perfusion (Table 1). Thus, the medium consumption is actually often greater with microfluidic perfusion cell culture than with conventional static cell culture.

### 2.2.7 Shear stress

In constant perfusion cell culture conditions the cells may be affected physically by exposure to shear stress. When dealing with simple rectangular cell culture chambers, the shear stress at the wall can be estimated as for the flow between two parallel plates by [15, 47]

$$\tau_s = \frac{6\mu Q}{Wh^2},$$
where \( \mu \) is the viscosity, \( Q \) is the flow rate, \( h \) is the chamber height and \( w \) is the chamber width. As seen from the equation, the shear stress increases as the dimensions of the chamber decrease and the flow rate and fluid viscosity increase. The impact of shear stress, reviewed by [47], vary depending on the cell type, but up to 0.65 Pa had no biological impact on most of the cells. At the chamber dimensions of the cell culture chips applied here and a flow rate of 500 nL/min, the shear stress has been estimated to \( 10^{-4} \) Pa [15], i.e. several orders of magnitude below those observed to effect shear stress on the cells. Thus, shear stress is not expected to have any effect on the cellular behaviour in the experiments performed in this project.

**Table 1 Overview of different dimensions/parameters of microscale to macroscale culture**

<table>
<thead>
<tr>
<th>Devices</th>
<th>Microchip</th>
<th>6 well plate</th>
<th>T75 flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of cell culture chamber</td>
<td>0.1 cm(^2)</td>
<td>10 cm(^2)</td>
<td>80 cm(^2)</td>
</tr>
<tr>
<td>Media volume</td>
<td>0.005 mL</td>
<td>2 mL</td>
<td>12 mL</td>
</tr>
<tr>
<td>Media height</td>
<td>0.5 mm</td>
<td>2 mm</td>
<td>1.6 mm</td>
</tr>
<tr>
<td>SAV</td>
<td>56.36 cm(^{-1})</td>
<td>6.12 cm(^{-1})</td>
<td>7.15 cm(^{-1})</td>
</tr>
<tr>
<td>Volume cell density at a seeding density of 100 cells/mm(^2)</td>
<td>200 cells/(\mu)L</td>
<td>50 cells/(\mu)L</td>
<td>67 cells/(\mu)L</td>
</tr>
<tr>
<td>Total culture volume per chamber in four days of cell culture of ASCs (at perfusion culture at 125 and 500 nL/min)</td>
<td>0.72 mL/2.88 mL</td>
<td>2 mL</td>
<td>12 mL</td>
</tr>
</tbody>
</table>
2.3 Stem cells

Stem cells have attracted enormous interest and attention because of their great potential. By definition, a stem cell has the capacity for self-renewal and the ability to differentiate into various functionally specialized cells [48]. Beyond embryonic development, stem cells are responsible for growth, homeostasis and repair of many tissues in the body throughout its life [26], such as for example homeostasis of the blood cells, the epidermis and the epithelium in the intestine. Besides providing information about what is regulating the fate of cells, stem cells have a great potential within regenerative medicine by repairing, replacing and/or regenerating damaged tissues. In addition, within the area of cell therapies, stem cells have the potential for replacing non-functional cells, as for example replacement of non-functional insulin-producing pancreatic cells in connection with diabetes. Gimble et al. [49] have suggested some criteria that should be meet for stem cells applied in regenerative medicine:

- Can be found in abundant numbers (millions to billions of cells)
- Can be harvested by a minimally invasive procedure with minimal morbidity
- Can be differentiated along multiple cell lineage pathways in a controllable manner and reproducible manner
- Can be safely and effectively transplanted to either an autologous or allogeneic host
- Can be produced in accordance with current Good Manufacturing Practice guidelines

However, investigation of stem cell behaviour and regulation of differentiation is complicated due to the fact that it is difficult to copy the in vivo microenvironment to an in vitro system which has the ability to support normal stem cell function.

2.3.1 Types of stem cells

As mentioned in the introduction to section 2.3, stem cells have the capacity for self-renewal and the ability to differentiate into functionally specialized cells. Stem cells can be divided into pluripotent and multipotent stem cells (Fig. 5) [48]. A pluripotent stem cell has the ability to divide infinitely and generate all the structures in the embryo, i.e. all the three germ layers (the endoderm, the mesoderm and the ectoderm) and the thereby derived cell types and tissues. Embryonic stem cells (ESC) (the inner cell mass of the blastocyst) are pluripotent stem cells. Due to the ethical concerns and limited availability connected to the use of embryonic stem cells, a method has been developed to induce the pluripotency of cells by introducing four defined transcription factors [50]. A multipotent stem cell is a committed stem cell (Fig. 5). It originates from one of the three germ
layers and is usually restricted in its differentiation to only cell types that are normally derived from the same germ layer as the committed stem cell. An example is mesenchymal stem cells (MSC) that originate from the mesoderm and have the ability to differentiate into cells of mesoderm origin, such as cartilage, bone, muscle and fat cells [51, 52]. A progenitor cell is a non-differentiated cell, but a cell that is committed to a specific differentiation pathway (Fig. 5). It is not capable of self-renewal and is therefore not considered as a stem cell [48]. The term ‘adult stem cells’ comprises various groups of committed multipotent stem cells, such as for example the hematopoietic stem cells, the neuronal stem cells, intestinal stem cells and the mesenchymal stem cells. They are all populations of embryonic-derived stem cells in the adult organism, and are localised in different so-called stem cell niches [48].

**Fig. 5. Simplified sketch of steps in cell differentiation.** A pluripotent stem cell has the ability to self-renew and differentiate into all cell types. A multipotent stem cell is a committed stem cell which has the ability to self-renew and differentiate into a limited number of cell types. A progenitor cell is an undifferentiated cell committed to a specific differentiation pathway. However, it is not able to self-renew and is therefore not a stem cell.

### 2.3.2 The stem cell niche

A stem cell niche regulates stem cell proliferation and differentiation [26]. A stem cell niche is comprised of the stem cells and the stromal support cells which interact with the stem cells. In addition, the niche contains ECM proteins, which provide structure, organisation and chemical/mechanical signals to the stem cells. A possible presence of blood vessels and nerve cells can convey systemic signals to the stem cell niche. Different niches exist for different adult stem cells, of which some of the most well-known are those located in the bone marrow for the hematopoietic stem cells and the bone marrow-derived MSCs, in the brain for the neuronal stem cells, at the base of the epidermis for the epidermal stem cells and at the base of the intestinal crypt for the intestinal stem cells [26]. In general, the stem cell niches support the development, maintenance and repair of the connected tissue.
2.3.3 Mesenchymal stem cells

The mesenchymal stem cells (or also named mesenchymal stromal cells) are a heterogeneous group of multipotent adult stem cells with a fibroblast-like morphology [51, 52]. They are isolated from the stromal fraction of many adult tissues (initially from the bone marrow stroma) and are defined by three criteria which as a minimum must be fulfilled [53]. First, they must be plastic-adherent when cultured in a standard cell culture dish/flask. Second, they must express and lack expression of specific surface antigens. And third, they must be able to differentiate into osteoblasts, chondroblasts and adipocytes in vitro. MSCs have received great attention, because of the lack of ethical concerns, wide availability and no immunological considerations compared to human ESCs. Although induced pluripotent stem cells (iPSC) are now available, epigenetic differences to human ESCs and the risk of tumour formation in the recipient still makes it controversial to use iPSCs in clinical trials [54]. Although bone marrow MSCs are the best described MSCs, low availability limits the use of those cells in clinical applications. However, another rich source of MSCs exists in the form of adipose-derived stem cells (ASC).

2.3.4 Adipose-derived stem cells

ASCs are isolated from liposuction aspirates, as first done by Zuk et al. [55]. The procedure is less painful for the donor and the yield is much higher compared to isolation of bone marrow stem cells (about 500-fold higher) [56]. The isolation procedure normally includes the following steps (Fig. 6) [55, 57, 58]: First, the lipoaspirate is washed several times to remove the majority of blood cells, which is then followed by a dispersion of the adipose tissue by collagenase digestion. After the digestion, the stromal vascular fraction (SVF) is isolated by centrifugation. The SVF forms a pellet, while the adipocytes and the lipids float in the supernatant. Besides containing ASCs, the SVF predominantly contains erythrocytes, leukocytes, endothelial cells and stromal cells. The erythrocytes are removed by using red blood cell lysis buffer. Often the ASCs have not been further purified except by the expansion of the cells on plastic surfaces (culture dishes/flasks) and thereby selection of the plastic adherent cells [55, 58]. The ASCs used in this project are provided by the Philippe Collas lab. In addition to the isolation procedure described above, the stromal cells from the Philippe Collas lab have been further purified by removing the endothelial cells (CD 31+) and leukocytes (CD45+) by magnetic cell sorting [57].

Like the mesenchymal stem cells in general, the ASCs are characterized by expression and lack of expression of certain surface antigens, and their ability to undergo adipogenic, osteogenic and chondrogenic differentiation [37, 59]. Besides the capacity to differentiate into adipocytes, osteoblasts and chondrocytes, the ASCs have been shown to undergo myogenic and neurogenic dif-
ferentiation [37]. Furthermore, ASCs have been reported to differentiate into endothelial and epithelial cells, hepatocytes, pancreatic cells and hematopoietic supporting cells [56, 59]. However, the differentiation of ASCs into cells of non-mesodermal origin is controversial [51, 60] due to a general lack of globally standardized methods for their isolation, purification, expansion and characterization. This lack of standardized methods complicates the evaluation and comparison of the different reported differentiation potentials.

Fig. 6. Overview of the steps which are normally included in the isolation procedure of ASCs. See the text for a detailed description.

There are many clinical applications of ASCs within the areas of adipose, bone and cartilage tissue regeneration/repair [52, 59, 60] due to the fact that they are relatively safe in use, easy to access and can be isolated in high numbers. The number of clinical trials rose from 9 in 2009 to 18 in May 2010 [59]. Because ASCs have been shown to display immunomodulatory activity, they have also been applied in for example treatment of graft-versus-host-disease, multiple sclerosis and rheumatoid arthritis. Furthermore, ASCs have been used in connection with bone tissue repair, cartilage regeneration in joint diseases and wound healing. Finally, there has been great interest within cosmetic surgery and soft tissue regeneration. Instead of direct fat-grafting, the ASCs have been used as a source of adipocytes [60].

2.3.5 Adipocyte differentiation

Fat tissue is a large organ that regulates energy homeostasis and works as an endocrine organ secreting many adipokines, which regulate, for example, insulin sensitivity, immune function and
lipid metabolism [61-63]. Fat tissue consists of either white adipose tissue or brown adipose tissue. The tissue is composed of cells of mesodermal origin that are able to accumulate large amounts of triglycerides in cytoplasmic vacuoles. Brown adipose tissue is mainly responsible for thermogenesis and is composed of brown adipocytes. They are characterized by many small lipid-filled droplets and a large number of mitochondria (Fig. 7). White adipose tissue is composed of white adipocytes, which in vivo are characterized by a single large droplet of stored triglycerides (Fig. 7). However, in vitro the fat is accumulated in many small droplets, which have a tendency to fuse during long-term cell culture [61]. The following presentation of adipocyte differentiation focuses on white adipocyte differentiation, because most fat in the adult human body is composed of white adipose tissue and because the white adipocyte differentiation process is the most interesting in connection with the use of ASCs within regenerative medicine. The literature is comprehensive and has been conducted within various cell types. Not all of it can be presented here; however, the most important parts, in particular in relation to the experimental work done in this PhD project, are pointed out and described in the following sections.

![Fig. 7. Types of adipocytes. MSCs differentiate into white or brown adipocytes. White adipocytes are characterized by a single large droplet of stored triglycerides. However, in vitro the fat is accumulated in many small droplets (immature white adipocyte), which have a tendency to fuse during long term cell culture. Brown adipocytes are characterized by many small lipid-filled droplets and a large number of mitochondria.](image)

The differentiation of multipotent stem cells into adipocytes is divided into two steps, like stem cell differentiation in general (Fig. 5) [61, 63, 64]. In the first step, the multipotent stem cells commit towards the adipogenic cell lineage and differentiate into preadipocytes. The preadipocytes cannot be morphologically distinguished from their precursor cells, but they are limited in their differentiation capacity to adipocytes only [63]. Upon exposure to adipogenic stimuli, the preadipocytes enter terminal differentiation to become functional adipocytes. During induction of terminal differentiation a transcriptional cascade is induced followed by expression of adipocyte specific metabolic genes and adipokines [61].

The adipocyte differentiation has mostly been studied in vitro in mouse preadipocyte models, such as 3T3-L1 and 3T3-F442A cells. These cell lines are isolated from mouse embryos and have
been selected for their ability to accumulate cytoplasmic triacylglycerol [64]. As a consequence, most studies have been concentrated around regulation of the terminal differentiation of preadipocytes into adipocytes, the subsequent activation of transcription factors and adipocyte specific genes, while less is known about the commitment step of multipotent stem cells towards the adipogenic cell lineage. The most commonly used multipotent cells are various human MSCs and the cell line C3H10T1/2, which is isolated from mouse embryos. The C3H10T1/2 cells have a fibroblast-like morphology and are functionally similar to mesenchymal stem cells [64].

Mesenchymal stem cells and preadipocytes proceed through adipocyte differentiation when cultured in growth medium added serum and a cocktail of adipogenic stimuli consisting of dexamethasone, isobuthyl-methylxanthine (IBMX), insulin and in some protocols indomethacin [37]. The mouse preadipocytes (3T3-L1 and 3T3-F442A) undergo one or two rounds of mitotic cell division upon induction of terminal differentiation, while C3H10T1/2 cells and human preadipocytes proceed through differentiation without cell division [63].

The following description of the knowledge about adipocyte differentiation is divided into three sections:

- Regulation of the commitment step towards the adipogenic cell lineage
- Regulation of the induction of the terminal differentiation into adipocytes
- Terminal differentiation.

### 2.3.5.1 Regulation of the commitment towards the adipogenic cell lineage

Little is known about the conversion of MSCs to committed preadipocytes; however some mechanical and molecular cues have been shown to be involved in the regulation. An overview is presented in Fig. 8.

High cell confluence (80-90%) has been reported to be required before induction of terminal differentiation by adipogenic stimuli [58, 65]. In addition, when cultured in a mixed adipogenic and osteogenic differentiation medium, high cell densities of human MSCs have been shown to promote adipogenesis, while low cell densities favoured osteogenesis [66]. McBeath et al. [66] further proposed that the initial plating density alone can affect the cell commitment and can do this separately from the downstream differentiation process. They showed that pre-culturing of MSCs in normal growth medium at low cell density inhibited later adipogenesis at both low and high cell densities, while pre-culturing at high cell density prevented later osteogenesis at both low and high cell densities. However, preculturing of MSCs at high cell density still required high cell density for efficient later adipogenesis. Furthermore, at normal cell maintenance the cells are regularly
passaged to low cell density, which according to our experiences does not prevent adipogenesis at a later high cell density condition. Thus, whether cell density determines stem cell fate and/or regulates/improves differentiation of preadipocytes is controversial.

Besides cell density, cell shape has been found to affect MSC commitment [66]. Cells grown in a mixed differentiation medium differentiated into adipocytes when grown on small islands (round shape) of pattern fibronectin, while cells grown on large islands (spread shape) differentiated into osteoblasts. The fact that the round cell shape promoted adipogenic differentiation correlates with the high cell density requirement, because the cells take up a more round shape when they are very confluent. The effect of cell confluence/shape on MSC adipogenic commitment has been associated with increased RHO-GTPase activity and thereby inhibition of actinomyosin formation [66].

Other parameters that have been observed to influence MSC fate decision is surface substrate stiffness. MSCs grown on polyacrylamid gels with different stiffness showed a larger proportion of cells differentiating into adipocytes at the lowest substrate stiffness compared to the highest substrate stiffness [67]. Another study [41] investigated the effect of micropost arrays with different rigidity on MSC fate decision. When culturing the cells in a mixed osteogenic/adipogenic differentiation medium, the high rigidity micropost array favoured osteogenic differentiation, while the low rigidity micropost array favoured adipogenic differentiation. In both studies [41, 67], the low stiffness/rigidity of the substrate correlates with a round cell shape and thereby further supports the suggestion that a round cell shape affects the MSC commitment towards the adipogenic cell lineage.

Regarding molecular cues, members of the transforming growth factor beta (TGFβ) superfamily, the bone morphogenic proteins (BMPs) BMP2 and BMP4, have been shown to be involved in the regulation of MSC commitment towards the adipogenic lineage [61, 64]. Treatment of multipotent C3H10T1/2 cells with BMP2 or BMP4, before exposure to the normal adipogenic differentiation protocol, promoted adipogenesis, while exposure to the differentiation protocol alone could not lead to adipogenesis [68].

Other molecular cues involved in MSC lineage commitment are the wingless-type mouse mammary tumor virus (MMTV) integration site family members (WNT) signaling molecules, which are cell secreted glycoproteins operating in a paracrine/autocrine manner in many developmental processes [61]. WNT10B has been shown to promote osteogenesis in MSCs through the canonical WNT signaling pathway, while inhibiting adipogenesis by suppressing expression of CCAAT-
enhancer-binding protein alpha (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ) [61, 69-71].
WNT10B binds to frizzled1 (FZD1) receptors and low-density lipoprotein receptor-related protein-5 or -6 (LRP5/6) co-receptors leading to stabilization of β-catenin, translocation of β-catenin to the nucleus and transcriptional activation of WNT target genes [72]. Likewise WNT5A inhibits adipogenesis by PPARγ inactivation. However, WNT5A signals through the non-canonical WNT pathway by binding to frizzled2 (FZD2) receptors [61, 69, 71, 72].

2.3.5.2 Regulation of the induction of terminal differentiation

Many studies of adipocyte differentiation have been performed in preadipocytes, which are committed to the adipogenic cell lineage, but which have not yet entered the terminal differentiation process into functional adipocytes. An overview of the regulation of the induction of terminal differentiation is presented in Fig. 8. Like in MSCs, WNT10B and WNT5A have been shown to inhibit adipogenesis in preadipocytes by reducing/inactivating PPARγ and C/EBPα [69, 72-74]. Accordingly, the prevention of WNT signaling induced preadipocytes to differentiate into adipocytes [73]. This correlates with IBMX as one of the adipogenic stimuli in the conventional differentiation protocol. IBMX is a cyclic adenosine monophosphate (cAMP) agonist, which has been shown to decrease expression of WNT10B by inhibiting cAMP response element-binding (CREB) binding to the WNT10B gene promoter [75]. Furthermore, adipocyte differentiation has been associated with the secretion of secreted frizzled-related proteins (sFRP) and Dickkopf-1 (Dkk1) [72]. sFRPs bind and sequester WNT10B [61, 72] and Dkk1 is an antagonist to LRP co-receptors, whereby they both inhibit WNT signaling [76]. In contrast to WNT10B and WNT5A, WNT5B has been observed to impede β-catenin nuclear translocation and thereby promote adipogenesis [77]. Thus, WNT signaling may be an important regulator of adipocyte differentiation through a cross-talk between mature adipocytes and adipose stem cells/preadipocytes, which further may be regulated by energy storage demands [72].

Members of the TGFβ superfamily have in addition to MSCs been shown to be involved in regulation of preadipocyte differentiation. TGFβ inhibits adipogenesis through SMAD3 signaling [61, 63, 78, 79]. Following phosphorylation, SMAD3 binds to C/EBPs and inhibits their transcriptional activity. Another member of the TGFβ superfamily, BMP2, increases differentiation in mouse embryonic fibroblasts (MEFs) grown in adipogenic medium [61, 80]. BMP2 signals through SMAD1, which mediate nuclear translocation of the transcription activator Schnurri 2 (SHN2) and thereby stimulate PPARγ expression.

Other transcriptional regulators of the induction of adipocyte differentiation are zinc-finger protein 423 (ZFP423) and transcription factor 7-like1 (TCF7L1) [61]. Both are expressed in adipo-
genic fibroblasts, but not in non-adipogenic fibroblasts. TCF7L1 acts downstream of the canonical WNT signaling pathway and promotes adipogenesis. ZFP423 is necessary for 3T3-L1 adipogenesis and can promote adipogenesis in non-adipogenic NIH-3T3 cells [81]. Finally, fibronectin and α5-integrin signaling have been reported to decrease differentiation of 3T3-F442A preadipocytes into mature adipocytes [61, 82, 83]. The fibronectin/α5-integrin signaling increases the levels of the active form of the RHO family GTPase RAC (RAC-GTP), which is normally inhibited during differentiation [83].

2.3.5.3 Terminal differentiation

A transcriptional cascade is activated upon addition of the adipogenic stimuli (IBMX, dexamethasone, indomethacin and insulin) to MSCs and preadipocytes, which ultimately results in expression of adipokines and adipocyte specific metabolic genes (such as leptin, adiponectin, lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4/aP2) and glucose transporter type 4 (GLUT4) [61, 63, 84]. Many transcription factors have been shown to influence the terminal adipogenesis process; however, by far the most important and central transcription factors are PPARγ, C/EBPα, C/EBPβ and C/EBPδ. PPARγ and C/EBPα have been shown to be present in about 60% of all adipocyte specific genes in mature adipocytes [85]. In general, the induction of terminal adipogenesis is a balance between positive and negative regulators of adipogenesis. An overview of the most important factors involved in the terminal adipogenic differentiation is described below and presented in Fig. 9.

C/EBPs

The transcription factors C/EBPβ and C/EBPδ are early markers of terminal differentiation [61]. C/EBPδ is induced by glucocorticoids (for example dexamethasone), while C/EBPβ is induced within a few hours after stimulation with cAMP agonists (for example IBMX) [86]. C/EBPβ expression is induced by activation of protein kinase A (PKA) and phosphorylation of CREB [61]. In addition, C/EBPβ expression is induced by Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling, and furthermore by the transcription factors KROX20 and Kruppel-like factor (KLF) 4 [61]. However, the initial stimuli of the JAK2/STAT3 signaling, KROX20 and KLF4 are unknown, but it could be insulin, some growth factors or BMPs [61]. Phosphorylation of C/EBPβ is required for DNA binding and happens through mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 beta (GSK3β) signaling induced by unknown stimuli [87]. Furthermore, glucocorticoids regulate C/EBPβ binding and acetylation [88], probably through the glucocorticoid receptor (GR) [89]. The early expression of C/EBPβ and
C/EBPβ leads to a later expression of C/EBPa, which is a direct transcriptional target of C/EBPβ [90]. However, C/EBPβ cannot induce C/EBPa expression in the absence of PPARγ [91].

**PPARγ**

PPARγ is the key regulator of terminal adipocyte differentiation [61, 63]. As the only factor involved in adipocyte differentiation, PPARγ has been shown to be necessary and sufficient for adipogenesis [92]. PPARγ exists in two isoforms, PPARγ1 and PPARγ2 [61]. PPARγ1 is the highest expressed of the two PPARγs in adipocytes, preadipocytes and MSCs, while PPARγ2 is adipocyte specific [93, 94]. However, both PPARγs promote adipogenesis equally well. Upon addition of adipogenic stimuli, the PPARG2 locus is dynamically and highly regulated by binding of the transcription factors C/EBPs, GR, STAT5 and retinoid X receptor (RXR) during an epigenomic transition state [61, 95, 96]. However, the specific dynamic DNA modifications are still unclear [61]. Furthermore, PPARγ and C/EBPs have been shown to regulate each other in a positive feedback mechanism [90, 95, 97]. The endogene PPARγ ligand is not yet known; however, transcription factor sterol response element-binding protein 1-c (SCREBP1c) and C/EBPβ increase PPARγ ligand production [98].

**Fig. 9. Overview of regulation of terminal adipocyte differentiation.** See the text for a detailed description.

**Other factors involved in terminal differentiation**

GATA-binding 2 (GATA2) and GATA3 inhibit PPARγ activation and must be repressed in preadipocytes for induction of adipogenesis [99]. Furthermore, many of the KLFs are involved in regulation of terminal differentiation [61, 63]. KLF4 induces C/EBPβ expression, while C/EBPβ
and C/EBPδ induce KLF5 expression, which further induces PPARγ2 expression [84]. In addition, KLF6 and KLF15 are pro-adipogenic [84], while KLF2 and KLF7 are anti-adipogenic [63, 84]. KLF2 directly inhibits PPARγ2 promotor activity [100]. Insulin promotes adipogenesis partly by enhancing SCREBP1c expression, which further increases the production of the PPARγ ligand [84]. Furthermore, insulin promotes adipogenesis by suppressing the inhibitory activity of forkhead transcription factor Fox01 [84].
Chapter 3

Materials and Methods

3.1 Fabrication

Design and fabrication of the microfluidic cell culture systems and components have been described in detail in [21] and papers attached this thesis as appendix 3 and appendix 4. System and system component fabrication have mainly been carried out by Peder Skafte-Pedersen and David Sabourin. However, fabrication of cell culture chips and µfluidic ribbons has routinely been carried out by the author of this thesis.

3.1.1 Cell culture chips

The employed microfluidic cell culture chips (Fig. 16) are made of poly(methyl methacrylate) (PMMA) (Plexiglas XT 20070, Röhm GmbH, Germany and Solaris Clear S000, PSC A/S, Denmark) and fabricated by micromilling followed by a UV assisted local heat bonding [101]. In short, the individual layers of the chip were cleaned with 70% EtOH before being exposed to UV (DY-MAX, 5000 EC with bulb 36970, CT, USA) for 90 s. Following exposure the layers were sandwiched between two glass slides in an alignment setup and bonded for 30 min in a laboratory press (PW 10 H, P/O/Weber, Germany) pre-heated to 90°C and at an initial applied pressure of approximately 8 kN for chips measuring 26 mm (w) x 76 mm (l) (Fig. 16b, c and d) and 12.50 kN for chips measuring 52 mm (w) x 76 mm (l) (Fig. 16a). The chips measuring 26 mm (w) x 76 mm (l) have a total thickness of 3.5 mm and the chips measuring 52 mm (w) x 76 mm (l) have a total
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thickness of 2.0 mm. Depending on the specific design, they are composed of individual sheets of PMMA ranging from 0.5 mm to 2 mm in thickness. The bottom layer of all chips is 0.5 mm for reduced optical path length from the sample to the objective. Inlets are spaced 2.25 mm apart to conform with 1536 micro titer well plate standards and placed along each chip side in groups of 8 to interface pumps and cell loading chips. The cell culture chambers for culturing of HeLa cells had a footprint of 1.5 mm (w) x 4 mm (l) capped by isosceles triangles, while the cell culture chambers for culturing of ASCs had a footprint of 1.5 mm (w) x 6 mm (l) also capped by isosceles triangles. Chamber height is 500 µm. For the chips measuring 26 mm (w) x 76 mm (l) the inlet and outlet channels are connected at the top surface of the chamber. For the chips measuring 52 mm (w) x 76 mm (l), the inlet are connected to the top of the chamber and the outlet to the bottom of the chamber.

3.1.2 µfluidic ribbons

The µfluidic ribbon (Fig. 11c) was monolithically cast from polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) inside a polycarbonate (PC) (Nordisk Plast, Denmark) mould. PC brackets were placed into the µfluidic ribbon moulds prior to PDMS casting and, during curing, integrated into the µfluidic ribbon. Mould pieces were aligned by 4 screws. An assembled µfluidic ribbon mould is shown in Fig. 11d. Before injecting PDMS into the mould, 240 µm optical fibres (Polymicro Technologies, Phoenix, Arizona) were threaded through 250 µm holes in the end pieces. PDMS was mixed in a 10:1 mass ratio of elastomer to curing agent and placed under vacuum to remove air bubbles. Mould vents allowed PDMS injection via a syringe and prevented air from being trapped in the finished part. The filled mould was placed in an oven at 60ºC overnight to cure. Once the mould had cooled enough to be handled, fibres were removed to yield integrated channels and the mould disassembled.

3.2 Cell culture and functional cell study experiments

3.2.1 Cells and cell culture medium

HeLa Tet-On® Advanced cells (631155, Clontech) were cultured in DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 10% Tet System Approved Fetal Bovine Serum (FBS) (631106, Clontech), penicillin 100 U/mL, streptomycin 100 µg/mL (P4333, Sigma), and geneticin (G-418) 100 µg/mL (11811-023, GIBCO). Adipose stem cells (ASC) (a gift from Philippe Collas, University of Oslo) were cultured in DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 15% v/v newborn calf serum (NCS) (N4762, Sigma), penicillin 100 U/mL, and streptomycin.
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100 μg/mL (P4333, Sigma). At conventional cell culturing the cells were incubated at 37°C and 5% CO₂.

3.2.2 General microfluidic cell culture

To avoid contamination of the cell culture by bacteria or fungi, all system preparations and changes of media reservoirs has been performed in a laminar flow bench and by the use of aseptic working procedures.

3.2.2.1 System assembling and priming

Liquid glass vials, caps, and silicone/poly (tetrafluoroethylene) (PTFE) tubing were sterilized by autoclaving before use. Glass vials or vial chips, tubing and connections to pumps were assembled onto the system base plate. The cell culture chip and tubes connecting the liquid reservoirs to the pumps or vial chips were filled separately with Milli-Q water to remove bubbles, before assembling the cell culture chip to the base plate (Fig. 17). Inlet and outlet reservoirs were coupled with PTFE tubing (BOLA 1810-10, Bohlender GmbH, Germany) and supplied with air supplemented with 5% CO₂ through a sterile filter (Fig. 19d). To avoid formation of gas bubbles a pressure of 0.3 bars was put on the flow system during the whole system preparation and cell culture period, only interrupted when for instance changing liquid or liquid reservoirs.

3.2.2.2 Sterilization of the fluidic network

The connected flow system was sterilized by flushing with 0.5 M NaOH (1.06498.1000, Merck) for 20-30 min at a flow rate of 4.5 μL/min followed by washing with sterile water (W3500, Cell culture tested, Sigma) for 30 min at a flow rate of 4.5 μL/min to remove all NaOH. At the sterilization, the reservoirs in the vial chip were completely filled with 0.5M NaOH, and at the following removal of NaOH washed three times with sterile water.

3.2.2.3 Cell culture chamber coating

Polyethyleneimine

The surface of the cell culture chambers was coated by passing a 50 μg/mL polyethyleneimine (PEI) (408727, Sigma) solution in phosphate buffered saline (PBS) through the chip at a flow rate of 4.5 μL/min for 15 min followed by 195 nL/min for 1.5 h at room temperature.

Fibronectin, laminin or collagen

The surface of the cell culture chambers was coated by passing the coating solution through the chip at a flow rate of 4.5 μL/min for 15 min followed by 195 nL/min for 45 min at 37°C. Fibronec-
tin (F0895, Sigma) was applied as a 20 µg/mL solution in PBS, laminin (L2020, Sigma) as a 20 µg/mL solution in PBS and collagen (C3867, Sigma) as a 40 µg/mL solution in sterile water.

After coating, the flow system was flushed with cell culture medium for 20 min at a flow rate of 4.5 µL/min or overnight at 33 nL/min before cell loading.

### 3.2.2.4 Cell loading

Prior to cell loading the outlet tubes were removed from the cell loading chip (Fig. 15d).

#### HeLa Tet-On® Advanced cells

HeLa Tet-On® Advanced cells were resuspended in cell culture medium added 0.025% w/v collagen (Sigma C3867) before approximately 10 µL suspension of a cell density of 5×10^5 cells/mL, corresponding to an expected surface density of 280 cells mm^2, was loaded into the embedded wells in the cell loading chip (Fig. 15f).

#### Adipose-derived stem cells

ASCs were resuspended in cell culture medium added 60% v/v NCS before approximately 10 µL cell suspension was loaded into the embedded wells in the cell loading chip (Fig. 15f). For system 1 (Fig. 10b) was used a cell suspension density of 1.8×10^5 cells/mL, corresponding to an expected surface density of 90 cells mm^2. Various cell suspension densities have been applied with system 2 (Fig. 10c).

At cell loading with system 1 (Fig. 10b), the system was transferred to the microscope and cells were introduced into the cell culture chambers by setting the pumps to run backwards at a flow rate of 4.5 µL/min for about 1 min. The flow was stopped when the entire chamber was filled with cells. At cell loading with system 2 (Fig. 10c), the cell loading was performed inside the LAF bench by activating a LEGO® Mindstorms flow program of 10 rotations in 6 sec (~ flow rate of 65 µL/min), which has been optimized to enable uniform cell loading.

After cell loading, the outlet tubings were attached to the cell loading chip again under aseptic conditions in a LAF bench (Fig. 15d and e). The cells were perfused with a low flow rate of 33 nL/min for 4 h to allow cell attachment. Following this attachment phase, the flow was change to the given cell culture perfusion rate.

### 3.2.2.5 Incubation

The microfluidic cell culture system was placed inside a conventional CO₂ incubator at 5% CO₂ and 37 °C (Fig. 18b). When performing time-lapse experiments, the microfluidic cell culture system was incubated inside an incubator (Incubator XL Dark S1, Zeiss) mounted a Zeiss life science
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microscope (AxioObserver.Z1) at 37 °C (Fig. 18c). To limit the influence of gas permeable materials on the cell culture medium, a flexible plastic cover was mounted the microfluidic cell culture system. The atmosphere inside the cover of the system was supplied with 8% CO₂ through a connected CO₂ module (CO₂ module S1, Zeiss). The percentage of 8% CO₂ has been optimized to ensure the maintenance of a pH of the cell culture medium inside the microfluidic cell culture system of 7.2 to 7.4.

3.2.2.6 Changes of liquid reservoirs and supply of medium during culture

Changes of cell culture medium or supply of fresh medium has been performed either by changing the glass vial or by suction of remaining medium in the vial chips followed by refilling of the reservoirs. Cell culture medium has been changed at least every 4 days.

3.2.3 Regulated fluorescent reporter gene expression

3.2.3.1 Plasmid creation

DD-ZsGreen1-DR was cut out from pZsGreen1-DR (632428, Clontech) and cloned in-frame with the DD tag sequence in the multiple cloning site in pTRE-Cycle2 (631116, Clontech). Plasmid amplification was carried out in One Shot® TOP10 Chemically Competent E. coli (C4040, Invitrogen) cultured either in LB Broth (L7275, Sigma) or on LB agar (L7025, Sigma) added Kanamycin 50 µg/mL (K1377, Sigma) or Ampicillin 100 µg/mL (171254, Calbiochem). The plasmids were purified by using PureLink™ HiPure Plasmid DNA Purification Kit (K2100, Invitrogen) and diluted in TE-buffer to 100 ng/mL.

3.2.3.2 Transfection of plasmid and regulated gene expression

HeLa Tet-On® Advanced cells passage 10 were loaded into the cell culture chambers on chip as described above. After 20 h of cell culturing and at a cell confluence of approximately 80%, the cells were transfected on chip. Briefly, 6.25 µg plasmid DNA (pTRE-Cycle2-ZsGreen1-DR) was diluted in 2.5 mL OptiMem medium (31985, GIBCO) and added 15.6 µL of Lipofectamine™ LTX (15338-100, Invitrogen) giving a proportion of DNA to Lipofectamine of 1:5. After gently mixing and incubation at room temperature for 25 min, the transfection complexes were diluted 1:3.2 in cell culture medium and then flushed into the cell culture chambers at a flow rate of 1.0 µL/min for 20 min. The transfection process was performed without flow for 60 min, followed by a short flow period at a flow rate of 0.5 µL/min for 10 min. These two steps were repeated for 6 h in total. Finally, the transfection complexes were removed by flushing with cell culture medium at a flow rate of 1.0 µL/min for 20 min. Cell culture was continued at a flow rate of 500 nL/min for another 18 h, before inducing mCherry and DD-ZsGreen1-DR gene expression by switching to cell culture me-
medium supplemented with doxycycline 0.5 µg/mL. DD-ZsGreen1-DR was stabilized by another switch to medium added doxycycline 0.5 µg/mL and Shield1 0.5 µM and perfusion with doxycycline and Shield1 was continued for another 21 h. Finally, a final switch was made to medium with doxycycline but without Shield1. Cell culture was continued for an additional 19 h before completion of the experiment.

3.2.3.3 Time-lapse imaging and image analysis of regulated reporter gene expression

The system was equipped with an atmosphere cover, clicked onto the microscope stage before reconnected to CO₂, pressure and electrical supplies. The system was incubated in an atmosphere setting of 37°C and 8% CO₂ to keep a pH of the cell culture medium at about 7.0-7.2. Time-lapse series of the regulated fluorescent reporter gene expression were recorded every hour by a Zeiss Axio Observer.Z1 microscope equipped with a 10x/0.3 Plan-Neofluar objective, Colibri LED light source and a Zeiss Axiocam MRm B/W camera. A scan of 6×2 images, all acquired with a z-stack of seven slices (5 µm between each slice), were recorded for each chamber. The DD-ZsGreen1-DR signal was acquired at 470 nm excitation wave length through a 62HE filter, while mCherry was excited at 555 nm and emission light acquired through a 43HE filter. DD-ZsGreen1-DR was exposed for 10 ms and mCherry for 500 ms. The images were processed by stitching the individual images together, converting the stitched images to one image and finally applying the AxioVision Extended Focus module on the z-stacks to obtain the best focused image. The fluorescent signal was quantified by calculating the densitometric sum (DS) based on the individual pixel values of the converted 16 bit image in the range between 1000-62955 for DD-ZsGreen1-DR and 1032-62439 for mCherry. Mean value of densitometric sum based on six individual chambers was calculated and normalized to the highest recorded value. The normalized averages and normalized standard error on the mean (SEM) for the regulated DD-ZsGreen-DR were plotted as function of time.

3.2.4 ASC differentiation experiments into adipocytes

3.2.4.1 Adipogenic differentiation media

All differentiation media has been freshly prepared just before use.

Adipogenic medium (AM)
Normal cell culture medium supplemented with IBMX (isobutyl-methylxanthine) 0.5 mM (I5879, Sigma), dexamethasone 1 µM (D4902, Sigma), indomethacin 0.2 mM (17378, Sigma), and insulin 10 µg/mL (I9278, Sigma).

AM diluted 4x
AM diluted 4 times in normal cell culture medium without serum.
1.5x AM + CM
AM prepared with 1.5 times the concentration of IBMX, dexamethasone, indomethacin and insulin compared to the normal concentrations in AM. The 1.5x AM is mixed 1:1 with conditioned medium (CM) (see below).

1.5x AM diluted 4x + CM
1.5x AM is diluted 4 times in normal cell culture medium without serum. The 1.5x AM diluted 4x is then mixed 1:1 with CM.

(1.5x AM + CM) diluted 4x
The 1:1 mixture of 1.5x AM + CM (1.5x AM + CM) is diluted 4 times in normal cell culture medium without serum.

3.2.4.2 Adipogenic differentiation in static cell culture
At a cell confluence of approximately 80-90%, the cell culture medium was changed to differentiation medium to induce the differentiation. The differentiation was continued up to three weeks with normally half of the differentiation medium changed every 3-4 days. However, in the static cell culture reference experiment to the perfusion cell culture experiments, the entire differentiation medium was changed every 4 days. As a negative control one flask was grown in normal cell culture medium.

3.2.4.3 Adipogenic differentiation in perfusion cell culture
Differentiation was induced at a cell confluence of approximately 80-90%. When testing differentiation at different cell densities, the differentiation was induced the day after cell loading. The cells were perfused with the different tested differentiation media (see above) and different tested flow rates. Medium reservoirs were exchanged with fresh medium every 4 days. For each differentiation experiment, one chamber was grown in normal cell culture medium as a negative control.

3.2.4.4 Collection of conditioned medium
In static cell cultures ASCs were grown to a cell confluence of approximately 80-90% before, the cell culture medium was changed to AM to induce the differentiation. Half of the conditioned differentiation medium was collected normally at day 4, 8, 12 and 16 after onset of differentiation and stored at 4 °C. The collections of CM from the 4 days during the differentiation period were pooled, aliquoted and stored at -20 °C.
3.2.4.5 Imaging and image analysis of ASC differentiation

Phase contrast images of ASC differentiation were normally acquired every second day by a Zeiss Axio Observer.Z1 microscope equipped with a 10x/0.3 Plan-Neofluar objective, and a Zeiss Axiocam MRm B/W camera. A scan of each cell culture chamber, all images acquired with a z-stack of 5 slices (6 µm between each slice), were recorded with a exposure time of 5 ms. The images were processed by applying the AxioVision Extended Focus module on the z-stacks to obtain the best focused image, stitching the individual images together and finally converting the stitched images to one image. Differentiation was measured by the total area of lipid-filled droplets using ImageJ and normalized to the total cell area at the start of differentiation. The lipid-filled droplets, which appear as white pixels, were marked by marking areas of two or more pixels with a gray value between 31347 and 65520. The area of cells at start of differentiation was measured by marking pixels with gray values of 12076 or more. Due to the very low contrast of ASCs, it was not possible to mark the whole area of each cell. However, the error was the same for all measurements.
Chapter 4

System Design Considerations
and Reliability

As described in the introduction, microfluidic cell culture has offered many new possibilities for in vitro cell culture experiments regarding control of the cellular microenvironment and, what is more, has improved the biological relevance by mimicking in vivo conditions more closely than by the use of conventional cell culture methods. Despite these achievements, microfluidic cell culture methods have not been widely implemented into biological/biomedical laboratories. Issues like requirement of skills not normally held by biologists, lack of scalability and difficulties with interpretation and comparison of results with previous knowledge obtained by conventional cell culture methods are obvious reasons for this lack of adoption by biologists. However, other probable explanations could be that the handling of such complicated devices is laborious and time-consuming. In addition, the devices may exhibit poor robustness and general reliability resulting in high failure rates.

In this chapter, microfluidic cell culture devices/systems are evaluated with regard to general usability within areas such as fabrication, assembling, portability, robustness, compatibility with standard laboratory equipment and sterilization procedures, and reliability. In line with the issues described above, the good performance as well as the less good performance will be presented and finally discussed in the last part of the chapter. The evaluation is based on working experiences acquired by the author through the practical use and testing of systems developed within the Pro-Cell project and the FAST group alongside the testing.
4.1 System requirements

To meet basic requirements for a microfluidic system for mammalian cell culture experiments, many aspects should be considered in the design and fabrication process. The most important requirements that should be met in order to provide a usable cell culture system are listed below:

**Fluidic actuation and control**

Control of the cellular microenvironment by means of fluidic actuation (for example pumps), control of the fluidic actuation (setting/changing of flow rate, turning on/off) and maybe valves (to selectively be able to open/close different microfluidic channels) should be provided. The cell culture compartment must be supplied with fresh cell culture medium containing nutrients and growth factors to support normal cellular functionality such as cell viability, proliferation, and differentiation. Likewise, cellular waste products have to be removed to maintain the optimal pH value of the medium. Finally, the fluidic control is a necessity to be able to perform spatial and temporal differential cell stimulation with various cues.

**Liquid reservoirs**

The volume of a microfluidic cell culture chamber is normally in the microlitre scale. However, dependent of the flow rate, a perfusion cell culture experiment consumes volumes of medium in the millilitre scale over a few days. For that reason, liquid reservoirs for fresh medium and waste medium, which are big enough to support at least 1-2 days of experimentation, are necessary.

**Liquid connections**

In order to provide a liquid line between medium/cues supply, cell sample, and waste, it is often necessary to have several liquid interconnections. The connections must be tight and robust to avoid leakage, bacteria contamination and introduction of bubbles into the liquid system. In addition, they should be easy to assemble.

**Cell culture chambers**

The central part of a microfluidic cell culture system is a chip with chambers designed to support cell growth and analysis. It should be possible to tailor the number and the design of the cell culture chambers specifically to different kind of assays.

**Biocompatibility**

When choosing materials and fabrication processes, it is essential to ensure that the materials and fabrication processes are not toxic to the cells or influence the general functionality of the cells.

**Compatibility with standard laboratory procedures**

In order to avoid contamination of the cell culture and to support cell adhesion, growth and differentiation, it is necessary that the device allows for sterilization (preferably autoclavage) of the
inner surfaces that will be exposed to the cell culture medium. In addition, the device should allow for surface coating procedures. Finally, functional cell analysis may require compatibility with cell transfection, immunochemistry, and other staining procedures.

**Compatibility with standard laboratory equipment**
For implementation of microfluidic cell culture systems as a tool in biological/biomedical research laboratories, the devices/systems must be compatible with existing laboratory equipment such as incubators, microscopes, or microplate readers.

**Optical properties**
A very important tool within biological/biomedical research is microscopy imaging, which implies a requirement for good optical properties of the cell culture compartment. It should be possible to achieve clear phase contrast and fluorescent images of the cells with low autofluorescence from the chip material.

**Scalability**
In a cell analysis study, normally, many conditions wanted to be analysed in the same experimental set up in a high-throughput manner. Therefore, one or two separate cell culture chambers are normally not enough for a common biological experiment. Often 10-30 (or even more) separate cell culture chambers are required. In addition, when designing a cell culture system that gives the possibility to analyse many samples at the same time, it is important to consider means to prepare and handle the cell culture system in a high-throughput manner not to make the experimental work very laborious and time-consuming.

**Portability**
Normally, a cell culture and analysis experiment involves several different working procedures, which take place at different locations, for example in a laminar air flow (LAF) bench, on a microscope stage or in an incubator. Accordingly, it is essential that the system easily can be transported between the work stations without interrupting the experiment.

**Usability**
In general, biologists do not have special physical and technical skills. For that reason, it is important that the ease of use has high priority. In addition, the time required to set up an experiment should be reasonable compared to the output.

**Reliability**
To facilitate low failure rates (< 10% in my opinion) and thereby reliable readouts, one of the most important requirements is reliability. The system and the components of the system must therefore exhibit a high degree of robustness to withstand the many operations of the cell culture system both during the experimental set up and during the run of long-term experiments.
4.2 Design approaches

When designing and fabricating a microfluidic cell culture device, the focus can be on the cell culture chip level or on the creation of a fully functional system. A very large part of the presented devices have been focused on only the chip level [4, 7, 29, 30]. Even though they display for example complex gradient generators or allow co-culture of different cell types, they require several peripheral components such as fluidic actuation in the form of macro-sized syringe pumps and liquid inlet/outlet reservoirs, thereby reducing the portability of the device. Other devices are based on passive perfusion and therefore do not require additional components [6, 9, 10]. One of the passive pumping principles rely on surface tension of small droplets to drive the fluid from the small droplet through a microchannel to a larger droplet [6]. The passive flow systems presented in [9] and [10] are based on gravidity driven flow. However, the inlet/outlet reservoirs contain only relatively small volumes and as a consequence have to be refilled once or twice a day. Another approach has been to construct fully integrated devices/systems, either modular [102, 103] or non-modular [5, 33, 104], with pneumatically controlled valves and pumps. Although presented as integrated systems, they usually still have liquid reservoirs as peripheral components, and thereby yet complicate transport between work stations.

Within the ProCell project and the FAST group the approach has been to design and fabricate a reliable, easy to use, transportable, reconfigurable modular microfluidic cell culture system with integrated pumps, valves, and inlet/outlet liquid reservoirs. A library of components has been developed (manuscript in preparation, Appendix 4) with the intention that the library forms the basis for the creation of many system variants tailored specifically to the application of interest.

4.3 System components evaluation

The developmental work regarding design and fabrication within the ProCell project and the FAST group has mainly been carried out by Peder Skafte-Pedersen and David Sabourin. The tested microfluidic cell culture devices/systems are illustrated in Fig. 10. The device shown in Fig. 10a was the basis for the developmental work during this PhD project. The first system version is shown in Fig. 10b and the second version in Fig. 10c. Both systems are modular and consist of various parts of the developed component library. The presented and evaluated components of the library are divided into pumps, motors and controllers, liquid reservoirs, fluidic interconnections, cell loading chips, and cell culture chips. The library also contains a valve; however, it has not yet been tested and optimized for cell culture experiments.
Fig. 10. Overview of tested devices/systems. a) A simple cell culture device with fluidic actuation by a syringe pump. Constituted the basis for the developmental work performed during this project. b) Microfluidic cell culture system, version 1, with up to 24 inlets. c) Microfluidic cell culture system, version 2, with up to 32 inlets.
4.3.1 Pumps

Like many microfluidic cell culture devices, the first tested cell culture device relied on a macro-sized pump (Fig. 11a) for fluidic actuation. This size of pump complicated transport of the device and the number of media inlets was very limited. In addition, the pump did not allow for pre-programmed change of flow rates, and due to long tubings it had a large dead volume. To solve these issues, a micropump has been developed and described by Skafte-Pedersen, Sabourin et al. 2009 [15, 21, 105]. The micropump (Fig. 11b) enables simultaneous peristaltic pumping of eight fluidic channels, and with a food-print of 30 mm (w) x 40 mm (l) x 20 mm (h) it can easily be integrated into a microfluidic system platform. As shown in Fig. 10b and Fig. 10c, three or four micropumps have been integrated into the microfluidic system platforms. The relatively low height of the micropump makes it compatible with the working distance between the stage and the condenser in a Zeiss life science microscope. The average standard deviation on flow rates between channels has been measured to 2.8% - 8.7%, and in addition, the pump has been shown to withstand more than 63000 rotations [15]. The number of rotations correspond to a volume of 33 mL or 45 days of constant perfusion at 500 nL/min [15], which is more than enough for a long-term cell culture experiment.

Fig. 11. Pumps. a) Macro-sized syringe pump. b) Assembled micropump. c) Parts of micropump. From left: Rotor bed, µfluidic ribbon, and multi-roller. d) Mold for casting of µfluidic ribbon. e) Precipitations in the cell culture medium after passage through the micropump.
As shown in Fig. 11c, the micropump consists of three parts: a rotor-bed, a µfluidic ribbon, and a multi-roller. The multi-roller is made of a central brass shaft, eight rolling stainless steel pins and end-pieces in aluminium, while the rotor-bed is milled in polycarbonate (PC). These two pump parts have been very robust, as the same parts have been used throughout the whole project. However, the µfluidic ribbon made in polydimethylsiloxane (PDMS) has displayed much less robustness and has accordingly often been replaced. The µfluidic ribbon is casted in PDMS into the mould together with the reusable end-brackets made of PC and fibres of 250 µm in diameter forming the microfluidic channels. The mould assembled with end-brackets, fibres and filled with PDMS is shown in Fig. 11d. After some practice, the fabrication of four ribbons took approximately 4-5 working hours. The elastomeric PDMS material in combination with the thin channels makes the ribbon quite fragile, which often has caused breaks on the ribbon both during fabrication and general use. When removing the very thin fibres after the casting, the risk of breaking the fibre inside the PDMS has been quite high. Finally, the introduction of even a small bubble along the channels at the casting resulted in a leaking pump. The µfluidic ribbon can withstand sterilization by ethanol, NaOH, and autoclavation. However, leakage at connections to chips has been observed after autoclavation. Regarding assembly, the micropump is easily put together by four screws and the ribbon connected to the cell culture chip or liquid reservoirs by two additional screws (Fig. 14). Normally, the end-brackets of the ribbon have been fixed in a small holder to facilitate easy liquid interconnections.

Besides the fragile character of the µfluidic ribbon, precipitations in the cell culture medium have been observed in the cell culture chamber in connection with pumping (Fig. 11e). The cause of these precipitations was investigated, and a summary of the results is shown in Table 2. To ensure that the precipitations were not already present in the medium, the medium was filtered immediately before pumping. However, precipitations were still observed. The influence of the micropump was further studied by comparing the amount of precipitations at different configurations (Fig. 12). A lot of particles were observed at forward flow, while almost no particles were observed at backward flow.

![Fig. 12. Overview of cell culture chambers, micropump, and flow directions. a) Forward flow with the cell culture chamber downstream of the micropump. b) Backward flow with the cell culture chamber upstream of the micropump.](image)
In order to investigate if the precipitations were caused by the PDMS material or the mechanical influence of the peristaltic micropump, the medium was drawn through a µfluidic ribbon before flowing into the cell culture chamber. No precipitations were observed, suggesting that the precipitations were caused by the mechanical impact of the peristaltic movement of the micropump or a combination of the mechanical impact and the PDMS material.

In addition to the cause of the precipitations, the source was examined by pumping either water, medium without serum, and medium with 10 % or 20 % serum. Pumping with water and medium without serum resulted in no precipitations, while the number of particles was shown to increase with higher serum content in the medium and higher flow rates. Different types of serum gave the same result. Furthermore, all precipitations were dissociated after flushing the chambers with trypsin-EDTA or 0.5 M NaOH, but not when flushing with PBS, again suggesting that the source of the precipitates is serum protein. Precipitation of serum protein could possibly affect cell functionality and was therefore tested by culturing and differentiating ASCs into adipocytes either by forward flow or backward flow. A negative impact on the three-week differentiation process was seen at flow rates of 125 and 500 nL/min, while no influence was noticed at low flow rates of 33 nL/min. This difference was due to the fact that almost no precipitations were observed at 33 nL/min, whereas many precipitations were observed at the higher flow rates. All tests were performed both on a completely new ribbon and a reused ribbon and no difference was demonstrated. In conclusion, the results suggest that the particles are precipitations of serum protein and caused by a mechanical impact of the peristaltic micropump.

Table 2

<table>
<thead>
<tr>
<th>Formation of precipitates</th>
<th>Pre-filtering</th>
<th>Forward pumping – high flow</th>
<th>Forward pumping – low flow</th>
<th>Backward pumping – high flow</th>
<th>Backward pumping – low flow</th>
<th>Drawing through ribbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medium - serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medium + 10% serum</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medium + 20% serum</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dissociation of precipitates</th>
<th>PBS</th>
<th>Trypsin-EDTA</th>
<th>0.5 M NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissociation</td>
<td>No effect</td>
<td>Complete dissociation</td>
<td>Complete dissociation</td>
</tr>
</tbody>
</table>

- No precipitates
+ . ++. +++ Indicates the degree of precipitates from few precipitates to many precipitates
Finally, PDMS has displayed unwanted properties like absorption of hydrophobic molecules [46, 106]. However, at differentiation of ASCs into adipocytes no difference has been observed with respect to degree of differentiation regardless of the micropump was upstream or downstream of the cell culture chamber. This issue is therefore not considered as a problem in the assays performed in this project.

### 4.3.2 Motors and controllers

Fluidic actuation has been provided either by a stepper motor (PRECIstep AM 1524 + 15A 1:152, Faulhaber) in combination with a custom-built control-box (Fig. 13a and Fig. 13b) and software or the LEGO® Mindstorms® MXT 2.0 robotics kit (Fig. 13c and Fig. 13d). The stepper motor provides flow rates of up to 5 µL/min and a smooth rotation with an average resolution ~ 20 pL per step [15], however the occlusion volume of the micropump is ~ 80 nL. The control system allows minimum flow rates of approximately 30 nL/min. Although the maximum flow rate of 5 µL/min is sufficient for most cell culture experiments, a uniform cell loading requires higher flow rates (see section 5.5.1). Even when increasing the viscosity of the cell loading medium, the cell loading still resulted in a gradient of cell densities over the length axis of the cell culture chamber.

![Fig. 13. Overview of motors and controllers. a) Stepper motor (PRECIstep AM 1524 + 15A 1:152, Faulhaber). b) Custom-built controller and software for flow control of the stepper motors. c) LEGO® Mindstorms® motor and controller. d) LEGO® Mindstorms® software for flow control.](image)

The size of the stepper motor is less than the micropump and the weight only 17.3 g [15], which facilitates easy integration into the microfluidic cell culture platform and keeps the system compatible with the working distance between the stage and the condenser in a Zeiss life science microscope. The motors have been robust in use, as only one motor has been replaced during the whole
Chapter 4

The custom-made pump control is based on Visual Basic for Applications (VBA) thereby preparing it for automated control through the Zeiss microscope control software AxioVision VBA module. However, a first version was developed by Felician Stefan Blaga and directly implemented in MS Excel. This first version only allows pump control, but not pre-programming of pump control, and has been employed for experiments throughout this project.

The LEGO® Mindstorms® MXT 2.0 robotics kit provides servo motors, a small controller, and a software interface (LabView®) for programming of the motors. The lowest rotational resolution is $1^\circ$, which makes the rotational movement less smooth compared to the stepper motor. The size of the motor is much bigger than the stepper motor (80.3 g, 11 cm (l) x 4 cm (w) x 4.5 cm (h)); however, it is still possible to integrate up to four micropumps and motors into the microfluidic cell culture system platform (Fig. 10c). Although the four motor configuration is possible, it does not fit into the microscope unless the two middle upright motors are removed before placing the system onto the microscope stage. The small size of the control box and the possibility for battery driven power supply makes the LEGO® Mindstorms® kit fluidic action and control more portable than the stepper motor control system. The pre-programming of the pumping is easily performed (even for a biologist) using the associated software with a LabView® interface. With the software it is possible to pre-program flow rates, breaks, or changes of flow rates over time. The LEGO® Mindstorms® motors have provided average flow rates from 33 nL/min to 30-40 µL/min [105]. The option to run at very high flow rates has made it possible to load cells at a uniform density over the entire cell culture chamber (see section 5.5.1). Although the LEGO® Mindstorms® kit has many advantages, some problems have been observed. The controller has three output ports that can be run at the same time. When running at high flow rates (~ 5 µL/min) from more than one port, the output has not been the same from both ports, probably due to too slow a response time of the control circuit. Furthermore, the motors move a bit while running, which can interfere with keeping the focus during time-lapse microscope imaging. Finally, it is important to ensure completely free space around the motor to avoid blocking of the motor rotation.

4.3.3 Liquid reservoirs

The liquid reservoirs are integrated into the microfluidic cell culture platform either in the form of glass vials or vial chips (Fig. 14a and Fig. 14b, respectively). The glass vials are placed into a holder, integrated into the microfluidic cell culture platform, and are connected to the micropump via poly (tetrafluoroethylene) (PTFE) tubings. The glass vials with polypropylene lids with silicon/PTFE septa and PTFE tubing all allow for sterilization by autoclavation. However, with a high number of liquid reservoirs, the initial assembling of the system and the change of cell culture me-
System Design Considerations and Reliability

dium during an experiment are quite time-consuming. The vial chip consists of a two layer bonded bottom chip with integrated fluidic channels, a block in the middle with eight wells of a volume of 3 mL, and a top lid. They are all made of poly (methyl methacrylate) (PMMA) to withstand NaOH, which is used for sterilization of the cell culture chip, also made of PMMA. Due to the fact that the vial chip is made of PMMA, it does not allow for sterilization by autoclavation or by ethanol. The inner surfaces are therefore sterilized by 0.5 M NaOH, making the sterilization process more complicated and time-consuming compared to autoclavation. The three parts are fixed together with screws and sealed at the bottom and the top via a gasket piece casted in PDMS. Changing of liquids can easily be performed by unscrewing the lid, removing the remaining liquid by suction and refilling with new liquid. However, the fixing of the lid to the well part by the six screws has to be done very carefully to avoid breaks in the well part. Furthermore, the fixing of the bottom bonded part to the middle well part with screws makes the bottom part very exposed to breaks in the bonding. The risk of breaks compared to a quite time-consuming fabrication is a disadvantage of the vial chips. Finally, increasing the number of required liquid reservoirs complicates the compatibility with microscopy imaging, due to limited space in the microscope between the stage, condenser and the built-in incubator cabinet (Fig. 18c).

Fig. 14. Liquid reservoirs and interconnections. a) Glass liquid reservoirs connected to micropump via PFTE tubings and a small gasket of a piece of silicon tube. b) Liquid reservoirs in the form of a vial chip. c) End-bracket of the µfluidic ribbon with integrated gaskets and ball joint interconnections casted in PDMS. d) Two micropumps with the one end of the µfluidic ribbon connected to the top of a cell culture chip and the other end connected to the bottom of a vial chip.
4.3.4 Liquid interconnections

The design of the ends of the µfluidic ribbon with gaskets and ball joint interconnections casted in PDMS (Fig. 14c) enables easy connection of eight microfluidic channels to either a cell culture chip, a liquid reservoir chip or another connection piece in one step (Fig. 14d). The connections are simply fixed by two screws from the top of the microfluidic platform (see also section 4.4) and have, in general, been very tight and robust. PTFE tubings connecting glass reservoirs to a microfluidic ribbon are sealed to a connection piece through a small silicone tube (Fig. 14a). Although these connections have been tight, they are not completely fixed and care must be taken not to detach the tubing.

The cell loading chip (Fig. 15c) is also based on the connection of eight fluidic channels in one step. For tight connection to the top of the cell culture chip, the bottom of the cell loading chip has integrated gaskets casted in PDMS. The channels/wells in the top of the cell loading chip are connected to the liquid reservoirs by PTFE tubing, which is pressed into the channels/wells in the cell loading chip (Fig. 15d and Fig. 15e). The connections are kept tight by applying a diameter of the PTFE tubing that is a bit larger than the diameter of the PDMS wells, thereby making a sealing. However, initially the connections were not fixed and leakages were observed.

4.3.5 Cell loading chips

The first version of a cell culture chip was made of PMMA with embedded micromilled wells 3 mm in diameter and 5 mm in height (Fig. 15a and Fig. 15b). The cell loading chip was connected to the cell culture chip through a small µfluidic ribbon and integrated as a part of the fluidic flow from inlet reservoirs to outlet reservoirs. The cell loading chip was connected to the liquid reservoirs through PTFE tubing pressed into the wells with a small silicone tube as a gasket (Fig. 15b). When performing the cell loading, the tubings were removed and the cell suspension loaded into the wells by pipetting. The cell suspension was drawn into the cell culture chambers by actuation of the micropump. However, it was very difficult to obtain a uniform cell loading, because of sedimentation of the cells during the relatively long pathway from cell loading chip to cell culture chamber. In addition, bubbles were easily introduced into the cell culture chambers, due to bubbles caught along the edges of the bottom of the milled wells.

The second version of a cell loading chip is made of PC with a middle piece casted in PDMS with embedded wells (Fig. 15c). It is placed directly on top of the inlet holes of the cell culture chip (Fig. 15d and Fig. 15e), thereby reducing the loading pathway, and as a result a more uniform cell loading is obtained. The wells, casted in the elastomeric material PDMS, allow for direct con-
connection to PFTE tubing (Fig. 15d and Fig. 15e) and in that way reduce the number of liquid inter-
connections compared to the first version of the cell loading chip. However, the PDMS is not that
robust, and care must be taken, when removing the PFTE tubing before cell loading, not to break
the PDMS casted insert. The cell loading is performed almost in the same way as with the first
version. However, to avoid introducing bubbles during the loading of the cell suspension into the
thin wells, the suspension is loaded by the use of a syringe and a 0.5 mm needle (Fig. 15f). Al-
though it is difficult to observe any bubbles through the relatively thick and unclear cell loading
chip, the cell loading has been performed without introducing any bubbles in most cases.

![Fig. 15. Overview of cell loading chips. a) First version cell loading chip connected to the cell culture chip through a small µfluidic ribbon. b) The wells in the first version cell loading chip were milled in PC. Connected to liquid reservoirs through PFTE tubing and a small piece of silicone tube as a gasket. c) Second version cell loading chip with eight wells embedded in a middle piece casted in PDMS. d) The cell loading chip is fixed directly on top of the inlets to the cell culture chip. Liquid connections to reservoirs are performed by pressing PFTE tubing into the PDMS wells. e) Cell loading chip assembled onto the cell culture chip and connected to reservoirs by PFTE tubings held together in a small collection piece. f) Loading of the cell suspension into the wells is carried out by the use of a syringe and a 0.5 mm needle. Images courtesy of Peder Skafte-Pedersen.]

Both PC and PDMS are compatible with sterilization by autoclavation. However, shrinking has
been observed after autoclavation of PDMS, and the inner surfaces of the cell loading chip have
therefore been sterilized by flushing with 0.5 M NaOH before assembling the system. Another
drawback regarding the risk of contamination is that the PFTE tubing has to be removed and re-
placed again to perform the cell loading. Although this is carried out in the LAF bench, it gives a
risk of contamination with bacteria or fungi.
4.3.6 Cell culture chips

The cell culture chips are made of three or four layers of PMMA bonded together by UV-assisted heat-bonding [15]. However, the UV-assisted heat-bonding should be carried out very carefully to be successful and, in addition, the bonding is not that robust resulting in many failures due to a broken bonding (Table 3). Regarding possible sterilization methods, autoclavation results in a bent chip due to too low a glass transition temperature of PMMA. PMMA in combination with the tensions applied to the material through the bonding is not compatible with sterilization with ethanol either. The ethanol causes breaks in the PMMA. Accordingly, 0.5 M NaOH has been used for sterilization of the inner surfaces.

![Cell culture chips diagrams]

**Fig. 16. Examples of microfluidic cell culture chips.** a) A 16 chamber chip, each chamber with two inlets. b) An eight chamber chip, each chamber with three inlets. c) An eight chamber chip, each chamber with one inlet. The channels are divided between three micropumps, thereby enabling application of three different flow rates in one experiment. d) A 24 chamber chip, each chamber with one inlet. The channels are divided between three micropumps, thereby enabling application of three different flow rates in one experiment. Images courtesy of Peder Skafte-Pedersen.

The design of the cell culture chip with respect to the number of cell culture chambers and routing of the microfluidic channels can be tailored specifically to each application. With the use of the first version microfluidic system (Fig. 10b) up to 24 inlets can be routed to the cell culture chambers in different ways. For example, 24 inlets can be routed to 24 separate chambers (Fig. 16d), 3 inlets routed to each of 8 chambers (Fig. 16b) or in principle 24 inlets routed to one chamber. Regarding the second version system (Fig. 10c) up to 32 inlets can be applied in the same way. An example of a cell culture chip for the second version system with 16 separate chambers each with two inlets is shown in Fig. 16a. The routing of more than one channel from different pumps to the same chamber enables flow from different liquid reservoirs at the same time, switching between...
different liquid reservoirs over time or creation of gradients of cues over time by differential flow rates. Furthermore, routing from different pumps to different chambers allows for different flow rates in the same experiment (Fig. 16c and Fig. 16d).

4.4 Systems evaluation

As presented in section 4.3 and Fig. 10, three microfluidic cell culture devices/systems have been applied/tested during this PhD project. The first device shown in Fig. 10a formed the basis for the developmental work, mainly performed by Peder Skafte-Pedersen and David Sabourin, resulting in the creation of the component library, from which the main components were presented and evaluated in section 4.3.1 to 4.3.6. The two microfluidic cell culture systems, shown in Fig. 10b and Fig. 10c, were built by the use of various components from the library. A detailed characterization of the system shown in Fig. 10b is presented in [107]. Assembling, portability, interference by bubbles and general reliability of the cell culture systems are presented and evaluated below in section 4.4.1 to 4.4.4.

4.4.1 Assembling

The principle of the assembling of the two microfluidic cell culture systems is the same. The basis is a system base plate, onto which various components are fixed by screws. The modular system and the fixation by screws make it easy to change a broken component without the need to fabricate a whole new system.

![Fig. 17. Overview of assembling of cell culture chip to system base plate. a) The cell culture chip is placed onto the small support frame in the bottom and the system base plate clicked onto the cell culture chip. The recesses of the system base plate fit exactly to the dimensions of the cell culture chip and the support frame. b) Finally, the system base plate is fixed to the cell culture chip and the support frame by screws from the top of the system base plate. Drawing courtesy of Peder Skafte-Pedersen.](image)
A complete system base plate consists of micropumps, motors, liquid reservoirs and liquid connections from the reservoirs to the micropumps. In order to assemble the cell culture chip to the base plate, the cell culture chip is placed onto a small support frame and the system base plate clicked onto the cell culture chip (Fig. 17a). The recesses of the bottom of the system base plate fit exactly to the dimensions of the cell culture chip and the support frame. Finally, the system base plate is fixed to the cell culture chip and the support frame by screws from the top of the system base plate (Fig. 17b). To obtain a bubble-free liquid network, it is normally necessary to fill the cell culture chip with water manually before assembling to the system base plate. A cell culture chip with a complicated channel and chamber network can be difficult to fill with water without any bubbles. Filling of the channels with CO₂ just before filling with water can help to obtain a bubble-free chip. The CO₂ is more easily dissolved in the water than atmospheric air. In addition to pre-filling the cell culture chip, the PFTE tubings, other liquid channels and the channels in the micropump are pre-filled with water by actuation of the micropumps. When connecting the system base plate and thereby the liquid channels to the channels of the cell culture chip, drops of water are created at both connection ends to get a connection without introducing any bubbles. The pre-filling of the system with water and the assembling of the system platform to the cell culture chip from the top by screws (Fig. 17) are very user-friendly and has in general resulted in a bubble-free liquid network after system assembling.

4.4.2 Portability

Normally a cell culture and analysis experiment takes place at many different locations, such as for example a LAF bench for system assembling, preparation and medium change (Fig. 18a), a cell culture incubator to maintain the required environment (Fig. 18b) and a microscope for imaging and cell analysis (Fig. 18c). Due to the integration of all components into a single system base plate, both systems are very easy to transport between the different work stations.

![Fig. 18. Portability enables easy movement between different work locations. a) Change of medium in LAF bench. b) Placed into an incubator for cell culture. c) Placed into a Zeiss life science microscope for imaging and cell analysis. Images courtesy of Peder Skafte-Pedersen.](image-url)
Especially the small controller of the LEGO® Mindstorms® MXT 2.0 robotics kit (Fig. 13c), which, in addition, allows for battery driven actuation of the micropumps, makes the transport very easy. The control system of the stepper-motors requires a small trolley for transportation (Fig. 13b). However, because the control system can easily be disconnected by unplugging the electrical wire at the system base plate, the transport of this first version system is still unproblematic.

4.4.3 Bubbles

Air bubbles are a common problem when working with microfluidic systems. They are unwanted, because they cause cell death, interfere with cell analysis and can affect the flow. The bubbles can be introduced into the system either during filling or assembling of the fluidic network. Furthermore, changes in pressure and temperature affect the amount of air that can be dissolved in water, which can lead to outgassing of air from the liquid in the fluidic system. Finally, air can be introduced during operation of the system through air permeable components, such as the µfluidic ribbon made of PDMS, which is a part of the peristaltic micropump. As shown in Table 3, section 4.6, air bubbles in the microfluidic system were a major problem during the first period of the system testing.

**Fig. 19. Bubbles and ways to avoid bubbles in microfluidic systems.** a) A bubble trap filled with bubbles at the inlet triangle. b) The same bubble trap as in a) six hours later and now filled with even more air. c) A cell culture chamber filled with air bubbles. d) Microfluidic cell culture system assembled with a pressure network. A T-piece at the inlet divides the air pressure into two, routing to the inlet and the outlet of the system. All glass vials are connected in series via PTFE tubings to the inlets and the outlets of the microfluidic system thereby applying the same pressure to both the inlets and the outlets of the microfluidic network. e) The inlet of the air-network is mounted with a 0.22 µm filter to avoid any contamination of the system. Image e) courtesy of Peder Skafte-Pedersen.
There are several ways to avoid bubbles in microfluidic systems. The liquid can be degassed before use and thereby be able to absorb more air during operation. However, this is not an option for microfluidic cell culture systems, due to the fact that the cells require O\textsubscript{2} and CO\textsubscript{2} in the cell culture medium to be viable. Passive bubble traps \cite{108} and combined bubble traps and bubble removal features \cite{109,110} have been demonstrated in the literature. In this project, a passive bubble trap was applied in the first part of the project (Fig. 19a and Fig. 19b). However for long-term cell culture experiments, the capture volume of the passive bubble traps was insufficient. Fig. 19a shows a bubble trap filled with air bubbles in the inlet triangle to the left. Six hours later, shown in Fig. 19b, the bubble trap was filled with even more air. The insufficient trapping volume finally resulted in cell culture chambers filled with air bubbles as shown in Fig. 19c. Other drawbacks of the bubble traps are that they make the fabrication process of the cell culture chip much more complicated and time-consuming and that they take up a lot of space of the already rather limited area of the cell culture chip.

To finally solve the problem of air bubbles destroying the cell culture experiments, the gas permeable characteristic of the PDMS components was exploited. The whole microfluidic system has been applied a pressure of 0.3 bar ~ 3 \times 10^4 \text{ Pa} of atmospheric air, added 5% CO\textsubscript{2} to maintain a proper pH of the cell culture medium. After applying pressure onto the microfluidic network, the microfluidic cell culture system has been operated successfully without introduction of any bubbles during up to three weeks of cell culture. The pressure is thought to cause the air to diffuse through the PDMS and thereby disappear. The same principle has successfully been used by Gómez-Sjöberg et al. \cite{5}.

In practice, the microfluidic network has been pressurized by applying atmospheric air added 5% CO\textsubscript{2} to all the inlet and outlet liquid reservoirs at a pressure of 0.3 bar ~ 3 \times 10^4 \text{ Pa}. The compressed air network has been created through PFTE tubing, which from one inlet tube was divided in two through a T-piece and then connected to either the inlet reservoirs or the outlet reservoirs. With respect to the glass vials, they were connected in a series via small pieces of PFTE tubing; however, if there were many glass vials, this assembling process could be rather time-consuming. This issue has been solved by the use of the vial chip, because the wells in the vial chip are connected through a channel-like recess in the lid of the vial chip. In this way, all the wells in the vial chip are pressurized by connecting one single PFTE tube to the lid of the vial chip. The connection to the lid is sealed by the use of a small piece of silicone tube as a gasket, but the connection was not fixed and care should be taken not to detach the PFTE tubing.
4.4.4 Compatibility with microscopy imaging

Both microfluidic cell culture systems are compatible with microscopy imaging using a Zeiss life science microscope (Axio Observer.Z1). The system base plates are designed to exactly fit into the recess on the microscope stage, originally made for fixation of well plates. In addition, it is possible for both systems to perform cell culture experiments with time-lapse microscopy imaging by the use of a microscope mounted incubator (Incubator XL Dark S1, Zeiss) and a CO₂ module (CO₂ module S1, Zeiss). However, due to limited space between the condenser and the microscope stage and the larger size of the LEGO® motors, it is only possible to run with up to two integrated micropumps and LEGO® motors with the second version microfluidic system.

4.4.5 System reliability

As described in section 4.1, general reliability is a very important requirement for a microfluidic cell culture system to be successfully implemented as a tool within biological/biomedical research. In Table 3 an overview is presented of the failures registered during operation of the tested devices/systems and the time consumed to set up an experiment. Each experiment is labeled by the date of the setting up of the experiment. The listed types of failures and parameters are described below:

**Broken chip bonding**
A broken bonding of the cell culture chip, at or near the microfluidic channels or cell culture chambers, results in a leakage in the microfluidic network, loss of pressure and finally introduction of air into the cell culture chambers.

**Broken µfluidic ribbon**
A leak in a µfluidic ribbon results in a leakage in the microfluidic network, loss of pressure and finally introduction of air into the cell culture chambers.

**Leakage at lid/septum of glass vial**
A leakage at the lid or the septum of one of the glass vials results in loss of pressure and finally introduction of air into the cell culture chambers.

**Leakage at cell loading chip**
A leakage at the liquid connections to the outlet chip results in a leakage in the microfluidic network, loss of pressure and finally introduction of air into the cell culture chambers.

**Broken cell loading chip**
The inner part of the cell loading chip is casted in PDMS with embedded wells for cell suspension loading. When removing the PFTE tubings to perform the cell loading as described in section 4.3.5, the PDMS part is very exposed to breaks, if not done carefully.
Broken vial chip – bottom part
The bottom part of the vial chip with microfluidic channels is a two layer bonded chip, which is fixed to the well part of the vial chip by screws. The tension applied to the bonded bottom part result in a high risk of breaking the bonding, giving rise to a leakage in the microfluidic network, loss of pressure and finally introduction of air into the cell culture chambers.

Broken vial chip
The bottom part and the lid are fixed to the well part of the vial chip by screws and embedded nuts. The fixing has to be quite tight to maintain the pressure; however this can cause the PMMA to break.

Other leakage
Other leakage can be leakages at liquid connections not listed above or a leakage resulting in a loss of pressure, for which the cause could not be found.

Contamination of microorganisms
Incidences of contaminations with either bacteria or fungi due to insufficient sterilization or aseptic handling during preparation and culture of the cells.

Chambers with bubbles during cell culture
Bubbles caught or formed during setting up of the experiment were normally removed before continuation of the experiment. The bubbles listed in this table are bubbles caught or formed during the running of the experiment.

Time spent to set up the experiment
The approximate time spent to set up the experiment in hours is listed.

Days of cell culture
The days of cell culture for each experiment are listed to be able to compare the failure rate to the duration of the experiment.

Errors during set up or run of the experiment
For each experiment is given if errors have occurred and the number of errors encountered. The errors are divided between during set up of the experiment and during run of the experiment.
### Table 3. Overview of failures during operation of the tested devices/systems

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<th>Errors during run of experiment</th>
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<th>Broken µfluidic ribbon</th>
<th>Leakage at lid/septum of glass vial</th>
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<th>Days of cell culture</th>
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* - Not relevant.  
* * In general, bubbles were frequently acquired during set up of the experiment and removed before continuing.  
* * The number in brackets is the number of errors. Bubbles in cell culture chambers are set to one error no matter how many chambers that were observed with bubbles.  
* * The given number is the number of experiments with bubbles caught/formed during run of the experiment out of the total number of experiments.
## Overview of failures during operation of the tested devices/systems continued

### System 1 pressurized

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<th>Experiment</th>
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<td>24</td>
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<tr>
<td><strong>Error rate in % of total numbers of experiments/samples</strong></td>
<td>44</td>
<td>39</td>
<td>33</td>
<td>33</td>
<td>17</td>
<td>11</td>
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<td>17</td>
<td>0</td>
<td>-</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

- Not relevant
- The number in brackets is the number of errors. Bubbles in cell culture chambers are set to one error no matter how many chambers that were observed with bubbles.
- The given number is the number of experiments with bubbles caught/formd during run of the experiment out of the total number of experiments.
### System Design Considerations and Reliability

#### Overview of failures during operation of the tested devices/systems continued

<table>
<thead>
<tr>
<th>System 2 pressurized</th>
<th>Errors during set up of experiment b</th>
<th>Errors during run of experiment c</th>
<th>Broken chip bonding</th>
<th>Broken μfluidic ribbon</th>
<th>Leakage at lid/septum of glass vial</th>
<th>Leakage at cell loading chip</th>
<th>Broken cell loading chip</th>
<th>Broken vial chip - bottom part</th>
<th>Broken vial chip</th>
<th>Other leakage</th>
<th>Contamination of micro-organisms</th>
<th>Days of cell culture</th>
<th>Chambers with bubbles during cell culture d</th>
<th>Time spent to set up experiment (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td></td>
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<td>x</td>
<td>x</td>
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<td></td>
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<td></td>
<td></td>
<td>22</td>
</tr>
</tbody>
</table>

| Error rate in % of total numbers of experiments/samples | 75 | 0 | 25 | 50 | 25 | 50 | 25 | 50 | 25 | 0 | 0 | 22 | 0/16 | - | 0 | - |

b The number in brackets is the number of errors. Bubbles in cell culture chambers are set to one error no matter how many chambers that were observed with bubbles.

c The given number is the number of experiments with bubbles caught/formed during run of the experiment out of the total number of experiments.
4.4.5.1 System failures

The system failures encountered when working with the first simple syringe pump device (Fig. 10a) and system 1 without pressure (Fig. 10b) were mainly observed during the run of the experiment, with a percentage of failed experiments of 57% and 100%, respectively (Table 3 and Fig. 20c). These high system failure rates during the run of the experiments were mostly caused by catching/formation of bubbles in the cell culture chambers (Table 3 and Fig. 20c). After setting pressure to the systems, the frequency of catching/formation of bubbles felt dramatically and thereby the system failures during the run of the experiments (Table 3 and Fig. 20c). The 13 earliest experiments with system 1 had a bubble error rate of 16%. However, no bubbles were observed during the run of the five latest experiments with system 1 and all four experiments run with system 2. In fact, the experiments have been performed without encountering any errors during the run of the experiments the last six months of the project (Table 3 and Fig. 20c). Notably, the decrease in bubbles observed in the cell culture chambers is inversely correlated to the number of days of cell culture, which increased from 4-6 days to 14-21 days (Table 3 and Fig. 20a).

![Fig. 20. Analysis of reliability. a) Time spent to set up the experiment and days of cell culture are shown in relation to the different tested devices/systems. b) The number of custom made parts each system consists of is related to the number of component errors during an experiment. c) The percentage of system failures during set up and during the run of the experiment as well as the fraction of chambers, which caught/formed bubbles during the run of the experiment, are related to the different tested devices/systems. n is the number of experiments.](image)
However, after setting pressure on the systems the percentage of experiments with errors during set up of the experiments increased from 14-22% to 44-75% of the experiments (Table 3 and Fig. 20c). The high percentage of experiments with errors during set up of the experiments was mainly due to broken system components, especially broken chip bondings and µfluidic ribbons (Table 3). Furthermore and not unexpected, the number of custom made system components, a system consists of, correlates with the number of component errors during an experiment (Table 3 and Fig. 20b). Contaminations of microorganisms in the microfluidic cell culture systems have not been observed, i.e. the materials and the design allows for efficient sterilization.

4.4.5.2 Time consumptions

The average time spent to set up an experiment without encountering any significant problems, was from 9-11 hours (Table 3 and Fig. 20a). Interestingly, the time spent to set up an experiment with the simple four chamber syringe pump device was also about 10 hours (Table 3 and Fig. 20a). Thus, the time consumption per chamber was 2.5 hours for the simple four chamber syringe pump device and 25 minutes for a 24 chamber complex system. However, the setting up of experiments with many incidents of errors caused by broken system components considerably increased the time spent to set up an experiment (Table 3 and Fig. 20a).

4.5 Summary

Table 4 is a summary of the advantages and drawbacks pointed out in the evaluation of the microfluidic cell culture systems and systems components in sections 4.3 and 4.4.

Table 4. Summary of system components and systems evaluation

<table>
<thead>
<tr>
<th>System components evaluation</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe pump</td>
<td>- No formation of precipitates</td>
<td>- The macro size complicates transport</td>
</tr>
<tr>
<td></td>
<td>- Robust</td>
<td>- Limited number of liquid inlets</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Large dead volume</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- No pre-programming of changes of flow rates</td>
</tr>
<tr>
<td>Micropump</td>
<td>- Simultaneous pumping of eight fluidic channels</td>
<td>- µfluidic ribbon quite fragile</td>
</tr>
<tr>
<td></td>
<td>- The small size allows easy integration</td>
<td>- Complicated fabrication of µfluidic ribbon</td>
</tr>
<tr>
<td></td>
<td>into a system platform</td>
<td>- Shrinkage observed at autoclavation</td>
</tr>
<tr>
<td></td>
<td>- Compatible with life science microscope</td>
<td>- The mechanical influence of the peristaltic pumping causes serum proteins in the cell</td>
</tr>
<tr>
<td></td>
<td>- Easy assembling of pump and liquid interconnections</td>
<td>culture medium to precipitate</td>
</tr>
</tbody>
</table>
### Summary of system components and systems evaluation continued

#### System components evaluation

<table>
<thead>
<tr>
<th>Motors and controllers</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stepper motor</strong></td>
<td>- Smooth rotation</td>
<td>- Flow rates no higher than 5 µL/min</td>
</tr>
<tr>
<td></td>
<td>- The small size of the motor makes it easy to integrate into system platform</td>
<td>- The highest flow rate not high enough for uniform cell loading</td>
</tr>
<tr>
<td></td>
<td>- Size of the motor compatible with microscopy imaging</td>
<td>- The size of the control elements requires a small trolley for transport</td>
</tr>
<tr>
<td></td>
<td>- Robust</td>
<td>- Currently not possible to pre-program changes in flow rates or breaks</td>
</tr>
<tr>
<td><strong>Lego motor</strong></td>
<td>- Placement of up to two motors compatible with time-lapse microscopy imaging</td>
<td>- Size larger than stepper motor, but still allows for integration into system platform</td>
</tr>
<tr>
<td></td>
<td>- Easy portable controller</td>
<td>- Less smooth rotation compared to stepper motor</td>
</tr>
<tr>
<td></td>
<td>- Possibility for battery driven power supply</td>
<td>- The controller has three output ports. However at high flow rates, the output has been observed not to be the same from all ports</td>
</tr>
<tr>
<td></td>
<td>- User-friendly controller software with LabView® interface</td>
<td>- The motor moves a bit while running. Can interfere with keeping the focus during time-lapse microscopy imaging</td>
</tr>
<tr>
<td></td>
<td>- Pre-programming of changes of flow rates and breaks possible</td>
<td>- The motor can be blocked if the space around the motor is not completely free</td>
</tr>
<tr>
<td></td>
<td>- Possible to run at very high flow rates (30-40 µL/min)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- The high flow rate possibility enables uniform cell loading</td>
<td></td>
</tr>
</tbody>
</table>

#### Liquid reservoirs

<table>
<thead>
<tr>
<th>Glass vials</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Allows for sterilization by autoclavation</td>
<td>- At a high number of inlet vials, the assembling and change of medium are quite time-consuming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Pressurising the system is time-consuming at a high number of liquid inlets</td>
</tr>
<tr>
<td><strong>Vial chip</strong></td>
<td>- Easy change of medium</td>
<td>- Cannot be sterilized by autoclavation</td>
</tr>
<tr>
<td></td>
<td>- A channel-like recess in the lid enables pressurising of the vial chip by connection of only one PTFE tube</td>
<td>- The fixing of the chip parts with screws makes the vial chip very exposed to breaks of the material or the bonding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Many liquid inlets complicate compatibility with microscopy imaging</td>
</tr>
</tbody>
</table>

#### Liquid interconnections

<table>
<thead>
<tr>
<th>Ball joint connections</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Easy connection of eight fluidic channels in one step</td>
<td>- µfluidic ribbon quite fragile</td>
</tr>
<tr>
<td></td>
<td>- Tight connections</td>
<td>- µfluidic ribbon complicated to fabricate</td>
</tr>
</tbody>
</table>
## Summary of system components and systems evaluation continued

<table>
<thead>
<tr>
<th>System components evaluation</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid interconnections</strong></td>
<td>- Eight connections made in one step</td>
<td>- The connections are not fixed and leakages have been observed</td>
</tr>
<tr>
<td><strong>Loading chip connections</strong></td>
<td>- Easy to load cell suspension into microwells by micropipetting</td>
<td>- Long loading pathway leading to cell sedimentation and uneven cell loading</td>
</tr>
<tr>
<td></td>
<td>- Easy cell loading by actuation of micropump</td>
<td>- Bubbles easily caught at the edges of the wells leading to bubbles in the cell culture chambers during cell loading</td>
</tr>
<tr>
<td></td>
<td>- Fewer liquid interconnections compared to the first version</td>
<td>- Difficult to observe if any bubbles are caught in the wells</td>
</tr>
<tr>
<td></td>
<td>- Easy cell loading by actuation of micropump</td>
<td>- The PDMS insert is fragile and very exposed to breaks when removing the tubings before cell loading</td>
</tr>
<tr>
<td></td>
<td>- Short loading pathway with less cell sedimentation. Enable uniform cell loading</td>
<td>- The narrow wells require cell suspension loading using a syringe and 0.5 mm needle, which are more complicated compared to micropipetting</td>
</tr>
<tr>
<td></td>
<td>- Fewer liquid interconnections compared to the first version</td>
<td>- Difficult to observe if any bubbles are caught in the wells</td>
</tr>
<tr>
<td></td>
<td>- Easy cell loading by actuation of micropump</td>
<td>- Shrinkage of the PDMS insert has been observed after autoclaving</td>
</tr>
<tr>
<td><strong>Cell loading chips</strong></td>
<td>- Good biocompatibility</td>
<td>- Complicated and time-consuming to achieve successful bonding</td>
</tr>
<tr>
<td></td>
<td>- Good optical properties</td>
<td>- Bonding very exposed to breaks</td>
</tr>
<tr>
<td></td>
<td>- Allows for adhesive and chemical free UV-assisted heat-bonding</td>
<td>- Low glass transition temperature of PMMA does not allow for sterilization by autoclavage</td>
</tr>
<tr>
<td><strong>Cell culture chips</strong></td>
<td>- The design of the cell culture chambers and routing of the channels can be tailored to the specific application</td>
<td>- PMMA in combination with tensions in the material does not allow for disinfection by ethanol</td>
</tr>
<tr>
<td><strong>PMMA chips</strong></td>
<td>- Routing of more than one channel to the same chamber enables flow from different liquid reservoirs at the same time, switching between different liquid reservoirs over time or creation of gradients of cues over time by applying differential flow rates</td>
<td>- Complicated and time-consuming to achieve successful bonding</td>
</tr>
<tr>
<td></td>
<td>- Routing from different pumps to different chambers allows for application of different flow rates in the same experiment</td>
<td>- Bonding very exposed to breaks</td>
</tr>
<tr>
<td></td>
<td>- Good biocompatibility</td>
<td>- Low glass transition temperature of PMMA does not allow for sterilization by autoclavage</td>
</tr>
<tr>
<td></td>
<td>- Good optical properties</td>
<td>- PMMA in combination with tensions in the material does not allow for disinfection by ethanol</td>
</tr>
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</table>
### Summary of system components and systems evaluation continued

<table>
<thead>
<tr>
<th>Cell culture systems</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Simple device</strong></td>
<td>- Simple assembling</td>
<td>- Low portability</td>
</tr>
<tr>
<td></td>
<td>- Low failure rate of components</td>
<td>- The percentage of cell culture chambers with formation/capture of bubbles during run of experiment high (50 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Limited scaling properties</td>
</tr>
<tr>
<td><strong>First version</strong></td>
<td>- Easy assembling of cell culture chip to system base plate</td>
<td>- Assembling of system easy but quite time-consuming</td>
</tr>
<tr>
<td></td>
<td>- Good portability</td>
<td>- Pressurized system leads to high failure rates of system components</td>
</tr>
<tr>
<td></td>
<td>- Pressurized system leads to low percentage of cell culture chambers with formation/capture of bubbles during run of experiment (11 %)</td>
<td>- Pre-programming of the motors currently not possible</td>
</tr>
<tr>
<td></td>
<td>- Compatible with microscopy imaging</td>
<td>- Controller elements require a small trolley for transport</td>
</tr>
<tr>
<td></td>
<td>- Compatible with time-lapse microscopy imaging</td>
<td>- The flow rate range is below 5 µL/min. Makes uniform cell loading impossible</td>
</tr>
<tr>
<td></td>
<td>- Scaling properties good with up to 24 parallel cell cultures</td>
<td>- Change of medium relatively time-consuming</td>
</tr>
<tr>
<td><strong>Second version</strong></td>
<td>- Easy assembling of cell culture chip to system base plate</td>
<td>- Assembling of system easy but quite time-consuming</td>
</tr>
<tr>
<td></td>
<td>- Excellent portability</td>
<td>- Pressurized system leads to high failure rates of system components</td>
</tr>
<tr>
<td></td>
<td>- Pressurized system lead to low percentage of cell culture chambers with formation/capture of bubbles during run of experiment (0 %)</td>
<td>- Pre-programming of the motors currently not possible</td>
</tr>
<tr>
<td></td>
<td>- Compatible with microscopy imaging</td>
<td>- Controller elements require a small trolley for transport</td>
</tr>
<tr>
<td></td>
<td>- Compatible with time-lapse microscopy imaging</td>
<td>- The flow rate range is below 5 µL/min. Makes uniform cell loading impossible</td>
</tr>
<tr>
<td></td>
<td>- Imaging with up to two integrated motors</td>
<td>- Change of medium relatively time-consuming</td>
</tr>
<tr>
<td></td>
<td>- User-friendly controller software</td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
<tr>
<td></td>
<td>- Pre-programming of motors possible</td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
<tr>
<td></td>
<td>- Easy change of medium</td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
<tr>
<td></td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
<tr>
<td></td>
<td>- Scaling properties good with up to 32 parallel cell cultures</td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Possibility for applying high flow rates enables uniform cell loading</td>
</tr>
</tbody>
</table>

### 4.6 Discussion

#### 4.6.1 Pumps

The developed micropump has many advantages over the conventional syringe pump, such as the small size and thereby easy integration into a system platform, and the simple one-step assembling and pumping of eight fluidic channels simultaneously. Originally, the µfluidic ribbon was meant as a disposable part of the micropump. However, as a result of the complicated fabrication
procedure, the µfluidic ribbons have been used until breaks or leakages in the ribbon are observed. This reuse has probably increased the failure rate, though breaks or leakages have also been noticed in new ribbons. A serious problem, in the form of precipitations in the cell culture medium, has been detected when pumping cell culture medium through the µfluidic ribbon. The precipitations were found to have a negative impact on the optical properties and the cell viability. Investigations showed that the particles were precipitations from the serum in the cell culture medium, which were formed due to the mechanical influence of the peristaltic micropump. Integrated peristaltic pumps made of PDMS have been employed in previously presented microfluidic cell culture systems [5, 104], so the precipitation problems were not expected. The inner dimensions (i.e. the surface to volume ratio), the way that the pump presses the channel and the occlusion volume may affect the tendency to form precipitates. The problem has currently been solved by running the pump in a backward direction with the cell culture chamber upstream of the micropump (Fig. 12b). However, to be able to switch between different pumps, and thereby to have the possibility of programmed cell stimulation with different cues and different concentrations over time, the configuration has to be forward flow with the cell culture chamber downstream of the pump (Fig. 12a and Fig. 16a and b). Work is currently going on in our lab to solve the problem. New materials and different inner channel dimensions are being investigated.

4.6.2 Motors and controllers

With respect to smoothness in rotation and size, the stepper motor is preferred to the LEGO® motor. The small size eases integration into the system platform and together with the rotational smoothness improves the compatibility with time-lapse microscopy imaging. The step-wise movement of the LEGO® motors can disturb the focus of the microscope. However, the low flow rate range (< 5 µL/min) of the stepper motor hinders uniform cell loading, which in connection with cell differentiation assays, for example, is an important parameter that has to be controllable. The LEGO® motor is favoured regarding the possibility of applying high flow rates, thereby enabling uniform cell loading, and the accompanying very user-friendly controller and software with pre-programming possibilities. However, signs that indicate that it is originally a kid’s toy have been noticed in the observation that the output from the three output ports on one controller is not the same at high speeds (actually a factor of two in difference). But this issue can easily be solved by using two separate controllers.

The ultimate goal of the ProCell project was to be able to perform conditional experiments. By means of automatic time-lapse microscopy imaging and image analysis, a signal should be sent to the pump controller and adjust the flow according to pre-programmed commands related to the
read-out of the image analysis. To enable these conditional experiments, the pump control system must be compatible with the Zeiss microscope control software AxioVision VBA module, which currently can only be fulfilled with the custom-made control system of the stepper motors.

Thus, none of the two tested motors meets all the requirements of an optimal motor. However, the LEGO® motor and the accompanying control system satisfy the most important requirements. Despite this, the LEGO® motor, to our knowledge, has not been utilized for fluidic actuation in other presented microfluidic cell culture systems outside the FAST group. Most fully integrated microfluidic cell culture systems presented in the literature utilize pneumatically controlled peristaltic pumps [5, 104], where the entire cell culture system, including the peristaltic pump, is casted in PDMS. However, the pneumatic control of micropumps usually requires more technical/physical skills than are normally held by a biologist.

4.6.3 Liquid reservoirs

Liquid reservoirs in the form of glass vials connected to the micropump by PFTE tubing are easy accessible and can be sterilized by autoclavage. On the other hand, a microfluidic cell culture system with many fluidic inlets and outlets, all connected to a glass reservoir, makes the assembling and change of liquid time-consuming and, furthermore, gives an impression of a very complicated system (Fig. 10b). In contrast, the application of the vial chip makes the overall impression of the system simpler and enables easy assembling and liquid change (Fig. 10c). However, the drawbacks of the vial chip are the fragile character caused by the design and fabrication procedure. The bonding of the bottom chip part is not robust and therefore very exposed to breaks when fixing the vial chip together with screws. In addition, the fixing of the lid part to the well part by screws and embedded nuts exposes the well part to breaks in the PMMA material. This can be solved by using longer screws and embedding of the nuts more deeply into the well part. Finally, sterilization by autoclavage is not possible.

In other presented microfluidic cell culture devices or systems the liquid reservoirs exist either as peripheral components [5, 7, 103, 111-113] or imbedded in the system in the form of small wells [6, 9, 10, 114]. A configuration with the liquid reservoirs as a peripheral component complicates transport, while the size of the embedded wells normally requires supply of medium once or twice a day, which is not user-friendly, especially not with long-term cell culture experiments. In a modular system approach, a vial chip, like the one tested in this project, is preferred. However improvements are needed with respect to robustness and sterilization possibilities. The optimal solution
would be a well part with embedded microfluidic channels, fabricated in one piece, and a lid part with a gasket, all fabricated in a material that allows for sterilization by autoclavation.

4.6.4 Liquid interconnections

The ball joint interconnection principle of the µfluidic ribbon allows, in one step, easy and tight connection of eight microfluidic channels from pump to cell culture chip, from pump to vial chip or from pump to a small connection piece for further connection to PFTE tubings (Fig. 14c and d). The connections from pump and cell loading chip to PFTE tubings are both based on press-fitting (Fig. 15b, d and e). The connections to the pump are not completely fixed, and therefore care should be taken not to detach the tubing. However, this has not been noticed as a problem. Liquid connections demonstrated in the literature are in general micro-to-macro connections, i.e. connections via tubings from chip/device to peripheral components such as pumps and/or liquid reservoirs normally by press-fitting [5, 7, 32, 103, 104, 113]. Through the system development in the ProCell project and the FAST group, the PFTE tubing connections have been eliminated by the development of the tubing-free vial chips at the connections to the micropumps, as shown in the second version microfluidic cell culture system (Fig. 10c). Likewise, the not unproblematic PFTE tubing connection to the cell loading chip (Fig. 15d and e) can be changed by the design of an additional vial chip fitting into the system platform at the cell loading chip location.

4.6.5 Cell loading chip

The development from the first version cell loading chip to the second version was based on a general requirement of as few liquid interconnections as possible and experiences that showed that the cell loading pathway should be as short as possible to avoid cell sedimentation, and thereby enable a uniform cell loading. By the use of the second version cell loading chip and the LEGO® motors for fluidic actuation, a uniform cell loading has been performed successfully (shown in section 5.5.1). However as described in section 4.6.4, the liquid connections from the cell loading chip to the liquid reservoirs have caused some problems in the form of leakages and, in addition, breaks in the PDMS well part insert, when removing the tubings before cell loading. Furthermore, it is difficult to properly sterilize the inner surfaces of the cell loading wells. When the PFTE tubings are pressed into the wells, as they are during the system sterilization by flushing with 0.5 M NaOH, the inner surfaces of the wells are not exposed to the NaOH solution. However, the change of the tubings to a vial chip would solve the connection problems as well as the sterilization problems. Solutions for cell loading presented in the literature are normally infusion/withdrawal from various, mostly not integrated, cell reservoirs by manual use of a syringe [32, 36, 113, 115, 116], or by peristaltic pumping/ differential pressure application [5, 7]. Currently work is going on in the
FAST group to develop a cell loading feature in the form of an integrated septum through which cells can be loaded manually by a syringe.

### 4.6.6 Cell culture chips

The modular system approach enables fast changes of the cell culture chip with respect to the chamber size and routing of channels, and thus provides the possibility of tailoring the design of the cell culture chip to a specific application. Regarding choice of material, complicated structures, pumps and valves can be fabricated relatively easily by the use of PDMS, and it is cheap and does not break [106]. Therefore, the majority of the devices/systems presented in the literature are made of PDMS. However, PDMS has displayed unwanted properties like absorption of hydrophobic molecules [46, 106], and due to the fact that many signaling molecules and drugs are hydrophobic, the decision within the ProCell project and the FAST group was to use PMMA as the material for fabrication of the microfluidic cell culture chips [15]. Furthermore, PMMA has been chosen as the material, because it has shown good biocompatibility [17, 18], good optical properties and in contrast to other materials it allows for adhesive-free and chemical-free bonding. The use of adhesives and chemicals may influence the biocompatibility. However, the employed UV-assisted heat-bonding has not been robust and reliable enough in use (Table 3). The rather low robustness in combination with a quite time-consuming fabrication process makes improvements with respect to the bonding process necessary. Work is currently going on in the FAST group to investigate other bonding procedures.

### 4.6.7 Microfluidic cell culture systems

In section 4.1 were listed the most important requirements that should be met to create a useful microfluidic system for mammalian cell culture experiments. But, do the tested microfluidic cell culture systems (Fig. 10b and c) fulfil these requirements? When evaluating a whole system, many aspects are related to specific components, which have already been discussed above in sections 4.6.1 to 4.6.6. However, overall, the requirement to develop a system with integrated fluidic actuation, liquid reservoirs, liquid interconnections and cell culture chambers, which at the same time displays good properties regarding optics, compatibility with standard laboratory equipment, portability and usability has been met. One missing feature is integrated valves to control the routing of the liquid network and thereby the cell manipulation. In the systems presented in this project, the routing from different inlets has been performed by switching between different pumps by turning on/off the pumps. Creation of concentration gradients over time can be performed by changing flow rates from two or more pumps over time. A valve has been developed in the FAST group
(Appendix 4), but it has not yet been tested into a microfluidic cell culture system. Biocompatibility and compatibility with standard cell culture procedures are considered in chapter five.

Regarding scalability, it is possible to perform up to 24 or 32 parallel cell cultures in one experiment, which in most cases is enough to investigate a biological issue. Notably, the scaling from a four chamber simple syringe pump device to a complex 24 chamber system did not increase the time spent to set up an experiment. In fact, the time spent to set up an experiment per chamber decreased from 2.5 hours to 25 minutes per chamber. It is important that scaling up the number of samples do not increase the time consumption more than reasonable, which for the system presented in this thesis has been shown to be possible. However, of course higher scalability, than shown here, would broaden the application’s possibilities, such as for example for drug testing.

The performance with respect to reliability must be improved to be satisfactory (Table 3 and Fig. 20c). As expected, the error rate due to component failures increases from working with a simple device to working with a more complex system. Thus, it is important to design a system as simple as possible, i.e. with as few components as possible. Furthermore, the many failures noticed during the experimental work with the second version of the cell culture system (Table 3 and Fig. 20c), may probably be due partly to teething problems, as only four experiments have been carried out in this project. The trend for the use of system 1 also shows more failures in the initial phase of the use of the system, suggesting that some practice is needed every time some components are changed or the system reconfigured. In the initial phase the user of course learns how to handle the components/systems, but also very importantly learns how to track errors.

By pressurizing the microfluidic cell culture system, the common problem of bubbles in microfluidics systems has been almost eliminated. Solving this bubble problem was very important, because no read-out was obtained as long as bubbles destroyed the cell viability, and in fact the systems were useless before setting pressure to the systems. However, the rise in component failures after applying pressure to the systems suggests that pressurization makes higher demand on the robustness of the system and system components. Even a small leakage is observed in a pressurized system. The leakage is observed already during set up of the experiment instead of during run of the experiment (in the form of bubbles). This change made it possible to run successful cell culture experiments with a biological read-out, see chapter 6. The weaknesses of the system components and the fabrication procedures has been discussed in sections 4.6.1 to 4.6.6 and most of the problems can be related to weak bondings (i.e. the fabrication method) and fragile µfluidic ribbons. In many cases the problems can be further related to the limited possibilities of fabrication methods and access to equipment during the developmental and prototyping process. Furthermore, most of
Chapter 4

the fabrication of for example cell culture chips, vial chips and µfluidic ribbons has been carried out by me (a biologist). A possible commercialization of the system by the industry, and thereby availability of ‘on the self components’, would provide much more advanced fabrication methods, more uniformity and robustness of the components, and thereby probably solve most of the reliability issues.

The usability issue in the form of the time-consumption to set up an experiment is partly related to the failure rate. A high system component failure rate significantly increases the time that is spent to set up an experiment and, in general, makes the time-consumption unpredictable. Normally, the time-consumption for conventional biological methods is quite well-known for a trained person, and this unpredictability is therefore very frustrating. In general, an average of 10 hours has been spent to set up an experiment of eight to 24 parallel cell cultures. This would normally have taken approximately 1 hour when performed as a static cell culture experiment in a well plate. To accept such a big difference as a biologist, it is obvious that the microfluidic cell culture system must provide some experimental possibilities that are not achievable by the use of a conventional static cell culture method, such as a more in vivo-like environment, co-culture possibilities and the study of paracrine and/or autocrine signaling. However, if new biological assays are possible, then, in my opinion, an experimental set up time of ten hours is reasonable and acceptable to a biologist.

The modular system approach improves the usability by making it easy to change a broken system component without the need to fabricate a whole new system. This is especially useful during the developmental process and prototype testing, where the fabrication possibilities are more limited. In addition, the modular approach allows for easy reconfiguration of the system.

Mammalian cell culture requires sterile laboratory utensils and work under aseptic conditions. A biologist is used to meet this requirement for sterile utensils by means of autoclavation, dry-sterilization or radiation, because it is easy and does not require any after-treatment before use, as for example sterilization by ethanol or NaOH does. Therefore, the ultimate goal for a microfluidic cell culture system would be that every component, exposed to the cell culture medium, allows for one of these sterilization methods. This goal is, however, difficult to fulfil, because many of the commonly-used materials do not withstand autoclavation or dry-sterilization, and due to the fact that a normal laboratory does not have access to sterilization by radiation.

4.6.8 Implementation of microfluidic cell culture into biological research

In the introduction of this chapter, it was questioned whether the lack of implementation of microfluidic cell culture into biological/biomedical research laboratories was partly due to issues like
poor usability, robustness and general reliability. The demonstrated performance of the microfluidic cell culture systems tested in this project makes these issues very probably one of the reasons for their lack of adoption by biologists. As with other microfluidic cell culture systems, the first version of the developed and tested systems in this project (Fig. 10b) has recently been published [107]. The publication describes the good performance of the system in the form of a detailed description of the physical properties and the system operation, and it demonstrates an application within regulated cellular gene expression. As shown in this chapter, the system performance has an additional dimension, which is not described in the paper. In general, this kind of information, such as the number of experimental tests, failure rates and time-consumption, is never shown in these device/system publications, although, this information is very relevant. The extent of the failures and problems, encountered during the testing of the systems in this project, is most probably similar to what is encountered in relation to other presented devices/systems in the literature. Many presented systems are much more complicated than the ones tested in this project and may therefore display even more problems and higher failure rates.

However, if the issues about the low robustness of the system components were solved, I would find the microfluidic cell culture systems, tested in this project, easy to use and acceptable to implement as a novel cell analysis tool. All the frustrations and the negative thoughts about the tested microfluidic cell culture systems, experienced during the project, have been related to bubbles (in the first phase of the project) and the low robustness of the system components.

4.7 Conclusion and outlook

The evaluation of the tested microfluidic cell culture systems, developed in the ProCell project and the FAST group, has shown that the requirement to develop a system with integrated fluidic actuation, liquid reservoirs, liquid interconnections and cell culture chambers, which at the same time displays good properties regarding optics, compatibility with standard laboratory equipment, portability and usability, has been met. Improvements regarding robustness and thereby reliability is shown to be needed, and these issues are probably one of the reasons for the lack of implementation of microfluidic cell culture systems into biological/biomedical research laboratories. Regarding the microfluidic cell culture systems tested in this project, the most important issues to be improved are the bonding of chips and the robustness of the µfluidic ribbon.

Based on experiences gained through the practical work in this project, general properties regarding the system design, as listed below, are suggested to be considered in addition to the requirements listed in section 4.1:
- The overall impression of the microfluidic cell culture system should be as uncomplicated as possible, so as not to ‘frighten away’ the biologist.
- The system and each system component should consist of as few sub-components as possible to reduce the failure rate.
- Much effort should be put into the creation of robust system components and thereby a reliable system.
- More efforts should be put into finding materials and fabrication methods that can withstand sterilization by autoclavation and thereby ease and reduce the time needed to prepare the system for cell culture experiments.
Chapter 5

Microfluidic Perfusion Cell Culture

Many practical issues must be considered when cell culture experiments want to be conducted into microfluidic perfusion cell culture systems. This project is only dealing with culture of adherent mammalian cells. The practical concerns involve assembling and priming of the system with liquid without introduction of air bubbles, sterilization of the inner surfaces of the liquid network and often surface coating of the cell culture chambers to allow/enhance cell attachment. Furthermore, the practical concerns include loading of the cells into the microfluidic cell culture chambers in a controllable manner and assessment of optimal flow conditions to support cell viability, proliferation and other cellular processes important for the study. Solutions to various practical issues are numerous, however some practical guidelines have been presented by Kim et al. [47].

Cell culture can be divided into cell culture maintenance and functional studies of the cells. This project has focused on establishment of procedures to be able to perform long-term functional studies of cells by the use of our developed microfluidic cell culture systems, while normal cell culture maintenance has been conducted by conventional cell culture methods. To avoid contamination of the cell culture by bacteria or fungi, all system preparations and changes of media reservoirs has been performed in a laminar flow bench and by the use of aseptic working procedures, which is normal procedure for conventional cell culture as well.
5.1 Cells

Diverse cell types behave differently with respect to for example surface attachment, proliferation and requirements to medium composition and renewal. Some cells require coating of the surface to enable or enhance cell attachment. Fast proliferating cells have a need for more frequent changes of cell culture medium to ensure sufficient supply of nutrients and removal of waste products. Thus, as in static cell culture the procedures for coating and medium renewal probably are cell type dependent.

Two different cell types have been employed in this project. HeLa cells (HeLa Tet-On® Advanced cells, Clontech) were used for initial establishment of the microfluidic perfusion cell culture procedures. In addition, the HeLa Tet-On Advanced cells, transient transfected with a fluorescent reporter plasmid, have been used as a model system to demonstrate the possibility of the microfluidic perfusion culture system to perform regulated differential gene expression of a gene of interest. In connection with the application of the microfluidic perfusion cell culture systems within stem cell research, adipose-derived stem cells (ASCs) have been cultured and induced to differentiate into adipocytes over a three week culture period.

5.2 Assembling and priming of the system

For assembling and priming of the microfluidic cell culture system is referred to section 4.4.1.

5.3 Sterilization

In order to avoid contamination of the cell culture by bacteria of fungi, it is necessary that the device is sterilized at the inner surfaces, which will be exposed to the cell culture medium. A biologist is used to sterilize by autoclavation or dry-sterilization, because it is an easy and biocompatible procedure, which does not require any after-treatment before use. Glass vials, PTFE tubings and vial lids were therefore sterilized by autoclavation before assembling. However, the PMMA used for fabrication of the cell culture chips, and as many of the materials generally used for fabrication of microfluidic systems, do not withstand autoclavation or dry-sterilization. The most commonly-used sterilization methods for microfluidic cell culture systems are therefore UV-radiation or flushing of the system with ethanol. However, PMMA exposed to tensions, as for example when bonded, cracks at contact with ethanol. Flushing with ethanol was therefore not an option for sterilization of our microfluidic cell culture system. Instead, 0.5 M NaOH was chosen as sterilization agent of the microfluidic network. Concentrations of 0.5 M to 1.0 M NaOH are commonly used to
clean and sterilize chromatography equipment [117]. The ability of NaOH to dissolve proteins has the additional effect of cleaning the system, when reusing system components.

After assembling and liquid priming of the system, the procedure for sterilization was flushing of the whole microfluidic network by 0.5 M NaOH at a flow rate of 5 µL/min for about 20-30 minutes, followed by a washing step with sterile water for at least 30 minutes to ensure removal of the NaOH.

![Image](image_url)

**Fig. 21. Effect of NaOH sterilization on HeLa cell viability.** The microfluidic system was flushed with 0.5 M NaOH for either 20 minutes or 2 minutes. As a reference the system was flushed with sterile water supplemented 1% penicillin/streptomycin for 20 minutes. Following perfusion with either NaOH or sterile water, the system was flushed with sterile water for 30 minutes, coated, and flushed with cell culture medium for 30 minutes before cell loading. 10x phase contrast images of the cells were acquired at day 1, day 2, and day 4.

To test the effect of NaOH sterilization of the microfluidic system on cell viability and proliferation, the system was flushed with 0.5 M NaOH for either 20 minutes or 2 minutes. As a reference the system was flushed with sterile water supplemented 1% penicillin/streptomycin for 20 minutes. Following perfusion with either NaOH or sterile water, the system was flushed with sterile water for 30 minutes, coated with polyethyleneimine (PEI) (see section 5.4.1), and flushed with cell culture medium for 30 minutes before loading of HeLa cells. As shown in **Fig. 21**, the sterilization by
0.5 M NaOH seemed to have no negative impact on cell viability and proliferation. Sterilization by NaOH has therefore been used at all microfluidic cell culture experiments performed during this project. The sterilization effect of 0.5 M NaOH has been satisfactory due to the fact that no contaminations of bacteria or fungi have been observed in a total of 38 conducted experiments.

5.4 Coating

As mentioned in the introduction to this chapter some cells may require a coating of the cell culture chamber surface with ECM proteins, such as fibronectin, laminin or collagen to allow or enhance cell attachment. Furthermore, many of the materials used to fabricate microfluidic cell culture devices are quite hydrophobic, which may interfere with cell attachment.

5.4.1 Coating for HeLa cell culture

Culture of HeLa cells has previously been performed in a microfluidic cell culture chamber made of PMMA in the FAST group [118, 119]. Satisfactory cell attachment was achieved by coating with polyethyleneimine (PEI) [119] or even without any coating [118]. PEI is a cationic synthetic polymer, which has been shown to enhance cell attachment of various cell types [120]. The effect of PEI coating on HeLa cell attachment was tested and compared to fibronectin coated PMMA surfaces. The cell culture chambers were either coated with PEI (50 µg/mL) for 1.5 hours at room temperature or fibronectin (100 µg/mL) for 10 minutes at 37 ºC, followed by flushing with cell culture medium for at least 30 minutes. After loading of the cells, the cells were allowed to attach at very low flow (33 nL/min) for four hours before changing to a flow rate of 250 nL/min. Coating of PMMA with fibronectin showed a slightly better and faster cell attachment compared to PEI (Fig. 22). However, PEI coating was chosen for coating at HeLa cell culture, because PEI is much cheaper, and because the HeLa cells were only used in the initial phase of establishment of microfluidic perfusion cell culture procedures and not for investigation of a biological question.

![Fig. 22. HeLa cell attachment in microfluidic perfusion cell culture at different coatings. Cells were cultured on PMMA coated with polyethyleneimine 50 µg/mL for 1.5 h or fibronectin 100 µg/mL for 10 min. 10x phase contrast images acquired 24 hours after cell loading. a) PMMA coated with PEI. b) PMMA coated with fibronectin.](image-url)
Coating at ASC culture

ASC culture on polystyrene surfaces in conventional cell culture in flasks or well plates does not require any coating of the surface prior to cell culture. To test ASC attachment on PMMA surfaces, preliminary tests were conducted on PMMA in static cell cultures. As shown in Fig. 23, cell attachment was very poor on PMMA without any coating (Fig. 23a) and on PMMA coated with PEI (50 µg/mL) (Fig. 23b), while a good cell attachment, similar to the reference on polystyrene (Fig. 23d), was observed on PMMA coated with laminin (10 µg/mL) (Fig. 23c). Thus, the preliminary results in static cell cultures suggested that a coating of PMMA with ECM proteins was needed to allow proper cell attachment.

![Fig. 23. Cell culture of ASCs on PMMA with or without coating in static cell culture. a) PMMA without coating. b) PMMA coated with PEI (50 µg/mL). c) PMMA coated with laminin (10 µg/mL). d) As reference polystyrene without coating. 10x phase contrast images acquired at day 1 after cell seeding.]

To further investigate the effect of different coatings with ECM proteins on ASC attachment, proliferation and differentiation in microfluidic perfusion cell culture, the cell culture chamber was coated with fibronectin (20 µg/mL), collagen (40 µg/mL), laminin (10 µg/mL) or not coated (flushed with PBS). This work was performed together with special course student Rasmus Finn Andersen. During coating, the chambers were flushed with the coating solution at a flow rate of 5 µL/min for 15 min followed by a flow rate of 200 nL/min for 45 min at 37 °C. After achievement of 70-80% cell confluence, the ASCs were induced to differentiate into adipocytes in adipogenic medium.
Fig. 24. ASC attachment, proliferation and adipogenic differentiation at different coatings of PMMA in microfluidic perfusion cell culture. The cell culture chambers were coated with fibronectin (20 µg/mL), collagen (40 µg/mL), laminin (10 µg/mL) or not coated (flushed with PBS). After achievement of 70-80% cell confluence, the ASCs were induced to differentiate into adipocytes in adipogenic medium. 10x phase contrast images acquired 4 hours after cell seeding. Cell attachment on PMMA coated with collagen a), fibronectin b), laminin c) and non-coated d). e) ASC proliferation. Measured by the percent of the area of the cell culture chamber covered by cells by using ImageJ. f) ASC differentiation into adipocytes. Number of cells with lipid-filled droplets counted manually over time after start of adipogenic differentiation. Data analysis done by Rasmus Finn Andersen.

A satisfactory cell attachment was observed with all coatings (Fig. 24). Even without any ECM coating the cell attachment was quite good (Fig. 24d). However, the fastest cell attachment was observed by coating with fibronectin (Fig. 24b). The ASC proliferation was almost similar at all coatings and without any coating (Fig. 24e), while large differences were seen with respect to differentiation efficiency (Fig. 24f). The differentiation efficiency was greatly reduced at fibronectin coating compared to laminin and collagen coating (Fig. 24f), which resulted in an almost similar differentiation efficiency. Likewise, less differentiation was observed at non-coated PMMA compared to laminin and collagen coated PMMA (Fig. 24f). Because similar cell attachment, prolifera-
tion and adipogenic differentiation was observed at laminin and collagen coating, and because collagen is cheaper than laminin, collagen coating was chosen as the standard coating procedure for the ASC adipocyte differentiation experiments performed in this project.

5.5 Cell loading

After assembling, priming the liquid network with water, sterilization of the inner surfaces and coating of the cell culture chamber surfaces, the cells have to be loaded into the chambers in a controllable manner without introducing bubbles into the liquid network. Two methods have been tested in this project. In preliminary experiments using the first simple microfluidic device shown in Fig. 10a cell loading was performed via direct infusion through the inlet tubing by the use of a syringe. However, it was very difficult to introduce the cells without introducing any bubbles, and bubbles are almost impossible to remove again at this point in the experiment without interfering with the cells or the sterility of the system. Another tested method, which has been employed in connection with both microfluidic cell culture systems developed in the ProCell project (Fig. 10b and c), is pumping of the cell suspension from a cell loading well into the cell culture chamber, as described in section 4.3.5 and Fig. 15. By this method, the cell suspension is loaded into a narrow cell loading well by a syringe and needle to avoid introduction of bubbles, whereupon the cell suspension is drawn into the cell culture chambers by activation of the micropump. After loading of the cells, the cells were, in general, allowed to attach at very low flow (33 nL/min) for four hours before changing to a flow rate supporting culture of the cells.

5.5.1 Uniformity of cell loading over the entire chamber surface

To obtain a uniform cell loading the flow of the cell suspension must be faster than the rate of cell sedimentation. However, by the use of system 1 (Fig. 10b) and thereby pump actuation by the stepper motors (Fig. 13a), with a maximum flow rate of 5 µL/min, it was not possible to obtain a uniform loading of HeLa cells (Fig. 25a). Another way to reduce the cell sedimentation during cell loading is to increase the viscosity of the suspension medium by, for example, addition of collagen [47]. To test the effect of collagen on the ability to obtain a uniform cell loading, HeLa cells were resuspended in cell culture medium (DMEM + 10% FBS + 1% penicillin/streptomycin) with different concentrations of collagen and loaded into the cell culture chambers by maximum flow rate (5 µL/min) using system 1. As shown in Fig. 25b, c and d, a concentration of 0.025% w/v collagen resulted in almost a uniform cell loading. Loading of HeLa cells has therefore been performed in normal cell culture medium with 0.025% collagen.
Fig. 25. Effect of increased cell suspension viscosity by collagen on HeLa cell loading. HeLa cells were resuspended in normal cell culture medium a) or cell culture medium with increasing concentrations of collagen, b) 0.005% w/v, c) 0.01% w/v and d) 0.025% w/v. 5x phase contrast images acquired immediately after cell loading.

With respect to cell loading of ASCs, the cell loading was likewise optimized by investigating the cell loading in increasing concentrations of serum (60%, 80% and 100%) in the normal cell culture medium (DMEM + 20% NCS + 1% penicillin/streptomycin). This work was performed together with special course student Rasmus Finn Andersen.

Fig. 26. Effect of increased cell suspension viscosity by serum on ASC loading. ASCs were resuspended in normal cell culture medium (20% NCS) or in normal cell culture medium with increasing concentrations of NCS (60%, 80% and 100%) and loaded into the cell culture chambers. 5x phase contrast images were acquired immediately after cell loading, and number of cells counted in three regions of the chamber by using AxioVision (Zeiss). Region 1 is the one third of the chamber at the outlet end, region 2 the middle third of the chamber and region 3 is the one third of the chamber at the inlet end of the cell loading. The number of cells is normalized to the number of cells in region 3 at the inlet end of the chamber. The error bars indicate standard error of the mean of 4 independent chambers in two independent experiments. The distribution of cells is shown for 20% NCS a), 60% NCS b), 80% NCS c) and 100% NCS d). Data analysis performed by Rasmus Finn Andersen.
None of the tested serum concentrations resulted in complete uniform cell loading (Fig. 26). However, an almost uniform cell loading was obtained in 60% serum (Fig. 26b) compared to 20%, 80% and 100% serum (Fig. 26a, c and d). The lower uniformity at 80% serum and 100% serum compared to 60% serum was probably due to clumping of the cells at the high serum content. Cell loading of ASCs has consequently been performed in normal cell culture medium with 60% NCS.

When increasing the chamber length from 4.4 mm to 6.6 mm, the loading of ASCs in 60% serum by the use of system 1 did not result in complete cell density uniformity (data not shown). However, the development of microfluidic cell culture system 2, with pump actuation by the LEGO® motors, offered the possibility to apply a much higher flow rate for the cell loading. By the use of the motor controller software LEGO® Mindstorms a flow program was optimized to enable uniform cell loading by pump actuation by the LEGO® motors. 10 rotations in 6 sec (~ flow rate of 65 µL/min) resulted in a uniform loading of ASCs, as demonstrated by loading of ASCs at six different cell suspension densities (Fig. 27).

Fig. 27. Cell loading by LEGO® motor pump actuation. Green fluorescent ASCs (infected with a lentivirus bearing a GFP expression cassette) at six different cell suspension densities were loaded into the cell loading wells by a syringe and needle (Fig. 15c and f) and pumped into the cell loading chambers by LEGO® motor actuation by 10 rotations in 6 sec (~ 65 µL/min). Cell loading uniformity shown at cell suspension densities of 0.6x10⁶ a), 1.6x10⁶ b), 2.6x10⁶ c), 3.6x10⁶ d), 4.6x10⁶ e) and 5.6x10⁶ f).

5.5.2 Controllability of actual seeding density

As demonstrated in the application of the microfluidic perfusion cell culture systems in stem cell research (chapter 6), it is not only necessary to be able to perform uniform cell loading over the
entire cell culture chamber. Besides uniformity, many functional cell studies, such as differentiation of ASCs into adipocytes (chapter 6), require an ability to control the actual seeding density. To compare the actual seeding density to the expected seeding density, ASCs, loaded at six different cell loading suspension densities by LEGO® motor pump actuation (Fig. 27), were counted immediately after cell loading. As shown in Table 5, only minor deviations (2-4%) from the expected cell seeding density to the actual cell seeding density were seen at the highest cell loading suspension densities of 3.6x10^5, 4.6x10^5 and 5.6x10^5 cells/mL. However, higher deviations (11-53%) were observed at the lower cell loading suspension densities, with a deviation of 53% at the lowest cell loading suspension density of 0.6x10^5 cells/mL. These results demonstrate that it is possible to control the actual seeding density in the cell culture chamber at high cell densities, whereas it is more difficult at low cell densities. The results in Table 5 are from one experiment.

Table 5.

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<th>Cell loading suspension density [cells/mL]</th>
<th>0.6x10^5</th>
<th>1.6x10^5</th>
<th>2.6x10^5</th>
<th>3.6x10^5</th>
<th>4.6x10^5</th>
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<td>80</td>
<td>130</td>
<td>180</td>
<td>230</td>
<td>280</td>
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<tr>
<td>Actual cell seeding density^b [cells/mm^2]</td>
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<td>103</td>
<td>177</td>
<td>223</td>
<td>290</td>
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<td>11%</td>
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</table>

^a Expected cell seeding cell seeding density calculated from the cell loading suspension density.

^b Actual cell seeding density determined by manual counting of the cells in 1 mm^2 in the middle of the chamber.

To further evaluate the cell loading controllability, the cell density of ASC cultures at onset of differentiation (1 day after cell loading) in two independent ASC differentiation experiments (Fig. 40 and Appendix 2) was determined in two parallel chambers for each of the three cell loading suspension densities. The cell number was determined by manual counting of the cells in 1 mm^2 in the middle of a cell culture chamber. To compare the cell density at day 1 to the expected seeding density, the cell density at day 1 is divided by a factor of 1.8, corresponding to an approximate division rate of ASCs the first 24 hours after cell seeding (Fig. 31b). As shown in Table 6, large variations are observed from experiment 1 to experiment 2 and within each experiment. These variations can be due to manual dilution errors or a different extent of cell sedimentation in the cell loading well before pumping in the cell suspension. In addition, the actual seeding density is, in general, higher than the expected cell seeding density, which further suggests a certain degree of cell sedimentation in the cell loading well before pumping the cell suspension into the cell culture.
chambers. Thus, the results indicate that it is not possible to satisfactorily control the actual seeding density, in particular not at the low cell densities.

Table 6.

<table>
<thead>
<tr>
<th>Variations in actual cell densities of ASC cultures at onset of differentiation</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Cell loading suspension density [cells/mL]</td>
<td>0.5×10^5</td>
<td>2.0×10^5</td>
</tr>
<tr>
<td>Expected cell seeding density^a [cells/mm^2]</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual cell density day 1^b Chamber 1 [cells/mm^2]</td>
<td>76</td>
<td>438</td>
</tr>
<tr>
<td>Actual cell density day 1^b Chamber 2 [cells/mm^2]</td>
<td>54</td>
<td>370</td>
</tr>
<tr>
<td>Estimated cell seeding density^c Chamber 1 [cells/mm^2]</td>
<td>42</td>
<td>243</td>
</tr>
<tr>
<td>Estimated cell seeding density^c Chamber 2 [cells/mm^2]</td>
<td>30</td>
<td>206</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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<tr>
<td>Actual cell density day 1^b Chamber 1 [cells/mm^2]</td>
<td>61</td>
<td>248</td>
</tr>
<tr>
<td>Actual cell density day 1^b Chamber 2 [cells/mm^2]</td>
<td>103</td>
<td>351</td>
</tr>
<tr>
<td>Estimated cell seeding density^c Chamber 1 [cells/mm^2]</td>
<td>34</td>
<td>138</td>
</tr>
<tr>
<td>Estimated cell seeding density^c Chamber 2 [cells/mm^2]</td>
<td>57</td>
<td>195</td>
</tr>
</tbody>
</table>

^a Expected cell seeding density calculated from the cell loading suspension density.
^b Actual cell seeding density determined by manual counting of the cells in 1 mm^2 in the middle of the chamber.
^c Estimated cell seeding density is determined by dividing the cell density at day 1 by a factor of 1.8, corresponding to an approximate division rate of ASCs the first 24 hours after cell seeding.

5.6 Cell culture incubation

For cell culture incubation, the microfluidic cell culture system was placed inside a conventional CO_2 incubator at conditions of 5% CO_2 and 37 °C (Fig. 18b), which ensured proper conditions with respect to temperature and pH of the cell culture medium. When performing time-lapse experiments, the microfluidic cell culture system was incubated inside an incubator (Incubator XL Dark S1, Zeiss) mounted a Zeiss life science microscope (AxioObserver.Z1) at 37 °C. To limit the influence of gas permeable materials on the cell culture medium, which at preliminary experiments was
observed to change the pH of the medium, a flexible plastic cover was mounted the microfluidic cell culture system. By a connected CO\textsubscript{2} module (CO\textsubscript{2} module S1, Zeiss) atmospheric air with 8% CO\textsubscript{2} was supplied to the covered microfluidic system. The percentage of 8% CO\textsubscript{2} had been optimized to ensure the maintenance of a pH of the cell culture medium inside the microfluidic cell culture system of 7.2 to 7.4.

5.7 Cell morphology and proliferation

Another important parameter regarding microfluidic perfusion cell culture is determination of the optimal perfusion flow rate. An optimal perfusion flow rate should support cell attachment, viability, normal morphology and cell proliferation. However, to restrict the consumption of reagents and avoid shear stress, the flow rate is wanted to be kept as low as possible. Due to the fact that different cell types behave differently with respect to cell division rate and display different sensitivities in \textit{in vitro} cell cultures, the optimal flow rate is probably cell dependent. Consequently, morphology and proliferation at different flow rates have been investigated for both cell types applied in this project.

5.7.1 HeLa cell morphology and proliferation

In microfluidic perfusion cell culture performed previously in the FAST group by Michael Stangegaard [17, 118], a flow rate of 1.67 µL/min in a 1.5 mm deep chamber, with inlet and outlet at the top of the chamber, was shown to support HeLa cell proliferation. This flow rate corresponded to an exchange rate of the medium in the cell culture chamber of 0.7/hour. The dimensions of the cell culture chambers used in this project for HeLa cell culture experiments were 4.5 mm (l) x 1.5 mm (w) x 0.5 mm (h). As a first approximation of a possible optimal flow rate, the flow rate applied previously by Michael Stangegaard was divided by a factor of three according to the reduced height of the cell culture chamber, which then corresponded to a flow rate of 500 nL/min. As follows, HeLa cell morphology and proliferation were investigated at four different flow rates, 125, 250, 500 and 750 nL/min, which correspond to an exchange rate of the cell culture medium in the cell culture chambers of 2.2, 4.4, 8.8 and 13.3 per hour, respectively. After cell loading, the cells were allowed to attach at very low flow (33 nL/min) for four hours before change of flow rate to 125, 250, 500 or 750 nL/min. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. As shown in \textit{Fig. 28}, the HeLa cells in perfusion cell cultures displayed similar morphology at all tested flow rates (\textit{Fig. 28a to d}) and a similar morphology to the static culture reference (\textit{Fig. 28e}).
Fig. 28. HeLa cell morphology in microfluidic perfusion cell culture at different flow rates. After cell loading and an attachment phase at a flow rate of 33 nL/min for 4 hours, the cells were perfused at four different flow rates. As a reference the HeLa cells were cultured in static culture in a conventional cell culture flask. 10x phase contrast images demonstrating HeLa cell morphology were acquired after 24 hours of culture at a flow rate of 125 nL/min. a), 250 nL/min. b), 500 nL/min. c), 750 nL/min d) and in static cell culture e).

However, with respect to cell proliferation, only perfusion at the two highest flow rates of 500 nL/min and 750 nL/min resulted in exponential growth of HeLa cells (Fig. 29b) with coefficients of determination $R^2$ of 0.993 and 0.999, respectively. Almost no cell division took place at flow rates of 125 nL/min and 250 nL/min, and many apoptotic cells were observed (data not shown). The cell doubling times at flow rates of 500 nL/min and 750 nL/min were 30.24 hours and 30.85 hours, respectively, whereas the cell doubling time in the static cell culture reference was 22.78 hours. Phase contrast images of cell proliferation at a flow rate of 500 nL/min at 24, 48, 72 and 96 hours after cell loading are shown in Fig. 29a.

Fig. 29. HeLa cell proliferation in microfluidic perfusion cell culture. HeLa cells were grown in DMEM +10% FBS+1% P/S in microfluidic perfusion cell culture for 4 days at four different flow rates, 125, 250, 500 and 750 nL/min, corresponding to an exchange rate of the medium in the cell culture chamber of 2.2, 4.4, 8.8, and 13.3 times per hour. a) 10x phase contrast images acquired 24, 48, 72 and 96 hours after cell loading. Cells cultured at a flow rate of 500 nL/min. b) HeLa cell proliferation in an area of 2 mm$^2$ at flow rates of 125, 250, 500 and 750 nL/min. HeLa cell proliferation in static cell culture shown as reference. Exponential growth fitting made by GraphPad Prism. The results are from two independent experiments, with flow rates of 125 and 750 nL/min from one experiment and flow rates of 250 and 500 nL/min from another experiment.
The results are from two independent experiments, with flow rates of 125 and 750 nL/min from one experiment and flow rates of 250 and 500 nL/min from another experiment. Thus, the results suggest that flow rates of 500 nL/min and 750 nL/min support normal cell morphology and satisfactory cell proliferation.

### 5.7.2 ASC morphology and proliferation

ASCs are slowly growing cells with a fibroblast-like morphology. As for HeLa cells, ASC morphology and proliferation were investigated at three different flow rates, 33, 125 and 500 nL/min, which correspond to an exchange rate of the cell culture medium in the cell culture chambers of 0.4, 1.5 and 6.0 per hour, respectively. The dimensions of the cell culture chambers used in this project for ASC culture experiments were 6.6 mm (l) x 1.5 mm (w) x 0.5 mm (h).

![Fig. 30. ASC morphology in microfluidic perfusion cell culture at different flow rates.](image)

After cell loading and an attachment phase at a flow rate of 33 nL/min for 4 hours, the cells were perfused at three different flow rates. As a reference the ASCs were cultured in static culture in a conventional cell culture flask. 10x phase contrast images demonstrating ASC morphology acquired after 24 hours of culture at a flow rate of 33 nL/min. a), 125 nL/min. b), and 500 nL/min. c) and in static cell culture d).

After cell loading, the cells were allowed to attach at low flow (33 nL/min) for four hours before no change or change of flow rate to 125 or 500 nL/min. The cells were cultured in DMEM supplemented with 20% NCS and 1% penicillin/streptomycin. As shown in Fig. 30, the ASCs in perfusion cell cultures had an almost similar cell morphology at the three tested flow rates (Fig. 30a to c) and a similar morphology to the static culture reference (Fig. 30d). However, at a flow rate of 33 nL/min more of very flat, spread cells were observed, whereas more elongated, slim cells were observed at 125 and 500 nL/min. However, both the much spread but still elongated morphologies and the long slim morphologies are observed at all three tested flow rates and in static cell cultures.

Regarding ASC proliferation, similar proliferation curves were observed at all three tested flow rates, and in addition, the proliferation curve for microfluidic perfusion cell cultures was similar to static cell cultures (Fig. 31). The approximate cell doubling time was 28-30 hours the first two days.
of cell culture, however, the division rate decreased after two days of cultures, when a certain cell density was reached. Phase contrast images of cell proliferation at a flow rate of 125 nL/min at 4, 24, 48, and 72 hours after cell loading are shown in Fig. 31a. The results are from one experiment. Thus, all three tested flow rates of 33, 125 and 500 nL/min supported satisfactory growth in microfluidic perfusion cell culture compared to static cell cultures.

![Fig. 31. ASC proliferation in microfluidic perfusion cell culture. ASCs were grown in DMEM+20% NCS+1% P/S in microfluidic perfusion cell culture for three days at three different flow rates, 33, 125, and 500 nL/min, corresponding to an exchange rate of the medium in the cell culture chamber of 0.4, 1.5, and 6.0 times per hour, respectively. a) 10x phase contrast images acquired 4, 24, 48, and 72 hours after cell loading at a flow rate of 125 nL/min. b) ASC proliferation in an area of 1 mm² at flow rates of 33, 125, and 500 nL/min. ASC proliferation in static cell culture shown as reference.](image)

### 5.8 Regulated gene expression

A type of functional cell study that fits well in with the possible read-out methods of our developed microfluidic perfusion cell culture system and, in addition, demonstrate the potential of the system is regulated gene expression of a protein of interest attached to a fluorescent reporter protein. The attachment of the protein of interest to a fluorescent reporter protein enables protein detection by real-time fluorescent microscopy imaging of living cells.

#### 5.8.1 Creation of a plasmid system for regulated gene expression

Thus, to demonstrate the ability of the microfluidic system to temporally regulate gene expression by external actuation, a plasmid-based system for regulated gene expression was set up. The pTRE-Cycle2 plasmid from Clontech consists of a bidirectional tetracycline sensitive promoter, which in the one direction encodes the red fluorescent reporter protein mCherry, and in the other direction encodes a DD signal peptide that tags a downstream protein for proteasomal degradation.
Chapter 5

The sequence encoding the green fluorescent reporter protein ZsGreen1-DR was inserted in-frame into pTRE-Cycle2 downstream to the DD tag, resulting in a plasmid named pTRE-Cycle2-ZsGreen1-DR. When this plasmid construct is transfected into HeLa Tet-On Advanced cells, which stably express the Tet-On transactivator, gene expression of the fluorescent reporter proteins can be regulated at two levels. Addition of doxycycline to the cell culture medium induces gene expression at the transcriptional level of both mCherry and DD-ZsGreen1-DR, but due to the upstream DD-tag DD-ZsGreen1-DR is quickly degraded. By addition of both doxycycline and Shield1, DD-ZsGreen1-DR is stabilized by Shield1 and thereby protected from proteasomal degradation. Thus, Shield1 regulate gene expression of DD-ZsGreen1-DR at the protein level.

Fig. 32. pTRE-Cycle2, a plasmid-based system for regulated gene expression. When pTRE-Cycle2-ZsGreen1-DR is transfected into HeLa Tet-On Advanced cells, gene expression can be regulated at two levels. Doxycycline induces gene expression of both of the fluorescent reporter proteins, mCherry and DD-ZsGreen1-DR. Due to the DD-tag, ZsGreen1-DR is quickly degraded unless Shield1 is added, which protects DD-ZsGreen1-DR from degradation.

5.8.2 On chip cell transfection

To enable the regulated gene expression by use of the created plasmid-based system described above in section 5.8.1, HeLa Tet-On Advanced cells were transfected in the microfluidic cell culture system. The cells were cultured for 20 hours after cell loading to a cell confluence of about 80% before the cells were transfected with pTRE-Cycle2-ZsGreen1-DR. The transfection complexes were diluted in cell culture medium and then flushed into the cell culture chambers at a flow rate of 1 μL/min for 20 minutes to ensure complete exchange of the medium in the cell culture chamber with medium containing the transfection complexes. The transfection process was performed without flow for 60 minutes, followed by a short flow period at a flow rate of 500 nL/min for 10 minutes to expose the cells to more transfection complexes. These two steps were repeated for 6 hours in total. Finally, the transfection complexes were removed by flushing with cell culture medium at a flow rate of 1 μL/min for 20 minutes. The cell culture was then continued at a flow rate of 500 nL/min for another 18 hours, before induction of mCherry gene expression by pump
switching to cell culture medium added doxycycline 0.5 μg/mL. Image of transfected cells acquired 24 hours after induction of mCherry gene expression shows a satisfactory transfection efficiency of approximately 50%.

5.8.3 Regulated gene expression by pump switching

The use of a cell culture chip with three inlets to each chamber (Fig. 16b) and with each of the inlets connected to a different pump, enabled switching of flow into the cell culture chamber from one inlet to another inlet channel by pump switching (turning off one pump and at the same time turning on another pump). This ability of temporal changing of culture conditions was used to demonstrate the potential of the microfluidic perfusion cell culture system for regulated gene expression experiments by the use of the created plasmid-based system described in section 5.8.1. The HeLa Tet-On Advanced cells were cultured and transfected with pTRE-Cycle2-ZsGreen1-DR on chip as described in section 5.8.2, and gene transcription of mCherry and DD-ZsGreen1-DR was induced by pump switching from an inlet with normal culture medium to medium added doxycycline 0.5 μg/mL. Regulated gene expression of DD-ZsGreen1-DR at the protein level was demonstrated by switching to medium with or without Shield1 0.5 μg/mL in addition to doxycycline. As shown in Fig. 34, the fluorescent signal from DD-ZsGreen1-DR gene expression increased after switching to medium with Shield1 (turning ON DD-ZsGreen1-DR gene expression) at approximately the same rate until it stabilizes at about 19-21 hours. After switching back to medium without Shield1 (turning OFF DD-ZsGreen1-DR gene expression), DD-ZsGreen1-DR was quickly degraded with a half-life of about 4.5 hours. This kinetics is similar to DD-YFP expression in NIH3T3 cells [121].
5.9 Discussion

Procedures for microfluidic perfusion cell culture have been investigated and established. The results show that it is possible to culture cells with a satisfactory cell attachment, morphology and proliferation. Furthermore, the potential of the system to perform temporal changes in cell culture conditions by pump switching has been demonstrated by regulated gene expression of a fluorescent reporter protein with real-time microscopy detection. However, some issues are to be discussed below.

5.9.1 Sterilization

0.5 M NaOH has been used for sterilization of the inner surfaces of the microfluidic network without any instances of contaminations. The sterilization procedure takes only about 1.5-2 hours in total (including washing to remove the NaOH). In addition, NaOH has the effect of cleaning possibly reused components due to the high solubility of proteins in NaOH solutions. For example, in connection with the precipitations of serum in the cell culture medium (section 4.3.1 and Table 2), 0.5 M NaOH was shown to completely dissociate/dissolve the precipitations. However, a disadvantage of the use of 0.5M NaOH is the high safety risk of NaOH. Although, the preparation of the system is always performed with gloves and behind a glass wall, a less hazardous agent may be
preferred. As mentioned in section 5.3, UV and ethanol are the most common employed sterilization methods for microfluidic cell culture systems. But as also mentioned, PMMA exposed to tensions is not resistant to ethanol, and due to the enclosed surfaces UV-radiation is not an option. Another possible sterilization agent could be 0.5% (w/v) hypochlorite, which has been used for sterilization of flow chambers for biofilm cultures [122]. However, they used a sterilization period of 3-4 hours, which is much more compared to the 30 minutes with 0.5M NaOH.

5.9.2 Cell loading

The approach to cell loading in our developed microfluidic cell culture systems has been withdrawal of the cell suspension from a cell loading reservoir into the cell culture chamber. This method reduced the risk of introduction of bubbles into the microfluidic network and also ensured that the same volume of cell suspension was introduced into each chamber in the cell culture chip. Furthermore, withdrawal by pumping reduced the path to the cell culture chamber and avoided passing of the cells through the pump. To achieve a uniform cell loading over the entire cell culture chamber, the flow rate has to be faster than the rate of cell sedimentation. In addition, the ability to obtain uniform cell loading depends on the length of the cell culture chamber; longer chambers makes it more difficult than in shorter chambers. Uniform cell loading was satisfactory obtained by resuspending the cells in cell culture medium added collagen or high serum concentrations to increase the viscosity of the medium (Fig. 25 and Fig. 26) and, furthermore, by applying a high flow rate of 65 µL/min for 6 seconds (Fig. 27). However, in spite of an increased medium viscosity, it was not possible by the use of system 1, with stepper motor actuation and a maximum flow rate of 5 µL/min, to achieve a uniform loading of ASCs in chambers with a length of 6.6 mm (2 mm longer than the chambers shown in Fig. 25) (data not shown). ASC culture experiments by use of system 1 have therefore been performed in a gradient of cell densities along the length axis of the cell culture chamber.

Another issue is the control of the actual seeding density of the cells, which for example has been shown to be an important parameter affecting adipogenic differentiation of ASCs (chapter 6). One cell loading experiment with green fluorescent ASCs showed satisfactory deviations of the actual cell seeding density to the expected cell seeding density at cell loading suspension densities of $1.6 \times 10^5$ cell/mL and higher (Table 5). However, the actual cell density of ASCs at onset of differentiation from some representative experiments showed large variations between the two shown experiments and compared to the expected cell density (Table 6). In general, the actual seeding density was higher than the expected, which indicate that uncontrollable cell sedimentation takes place in the cell loading wells in the period from loading of the cells into the wells until actuation.
of the pump. Eight wells have to be loaded before the pump can be actuated to load the cells into the cell culture chambers. Methods used in other microfluidic cell culture devices are infusion/withdrawal from various, mostly not integrated, cell reservoirs by manual use of a syringe [32, 36, 113, 115, 116], or by peristaltic pumping/differential pressure application [5, 7]. However, the actual detailed performance of the cell loading methods is not presented. But a method employing direct cell loading by a syringe probably enable better control of the actual seeding density due to less cell sedimentation before loading of the cells into the cell culture chambers.

5.9.3 Cell culture incubation

For incubation of the cell culture, the entire microfluidic cell culture system was placed inside a conventional CO\textsubscript{2} incubator. A microfluidic cell culture chamber previously developed within the FAST group [16] had an integrated indium-tin-oxide heater, which enabled cell culture outside the incubator. However, the embedded heater complicated the cell culture chip fabrication and reduced the optical properties of the cell culture chamber. Consequently, the microfluidic cell culture system developed in the ProCell project was decided not to have an integrated heater. Due to the design of the system with integrated pumps and liquid reservoirs, the incubation inside a conventional incubator or incubation in the microscope for time-lapse studies has been completely unproblematic. Furthermore, the cell culture chip fabrication has been much simpler without any integrated components.

5.9.4 Cell proliferation

Satisfactory proliferation curves of HeLa cell and ASC culture in our microfluidic perfusion cell culture systems were observed compared to the curves for static cultures of HeLa cells and ASCs (Fig. 29 and Fig. 24). However, the results show that the optimal flow rate is cell dependent, with the faster dividing HeLa cells requiring a higher flow rate to support cell division than the slower dividing ASCs. Young et al. [1] have presented a way to make a theoretical estimate of the optimal perfusion flow rate in terms of a critical perfusion rate (CPR) (see also section 2.2.5, equation (9)). According to their suggestions, CPR can be estimated by the length of the cell culture chamber divided by the effective culture time (ECT) in microscale culture. ECT is the time between changes of the cell culture medium for the respective cell type, and ECT in microscale culture can be scaled from ECT at macroscale cell culture according to the height of the cell culture chamber [1]. For HeLa cells with a usual interval between changes of medium of 48 hours in macroscale culture with a media height of about 1.5 mm, ECT in microscale culture with a chamber height of 0.5 mm is about 16 hours. At a length of the cell culture chamber of 6.6 mm, CPR is then estimated to 1.15\times10^{-7} \text{ m/s}. With a chamber height of 0.5 mm and a chamber width of 1.5 mm, the correspond-
ing perfusion flow rate is 5 nL/min. However, the results presented in Fig. 29b showed that flow rates of 125 and 250 nL/min did not support HeLa cell division, while flow rates of 500 and 750 nL/min supported an exponential growth of the HeLa cells similar to the HeLa cell division in static cultures. As mentioned in section 2.2.5, the presented theoretical estimation of the optimal perfusion flow rate is based on very rough assumptions. Firstly, they assume that ECT is equal to the complete consumption (reaction) of a given molecule with the cells. However, at conventional cell culture, the time between changes of cell culture medium for a given cell type is normally not experimentally determined, but in general based on recommendations from the supplier or personal experiences/opinions. Furthermore, the presented model assumes that the time scale of diffusion is not limiting the accessibility of molecules. But, in perfusion culture the diffusion time across the chamber is normally slower than the convection time in the perfusion direction, i.e. the molecules in the upper layer will probably just flow through the system. Finally, different molecules in the cell culture medium needed for cell division probably have very different reaction time scales with the cells. Our results suggest that experimental determination of the optimal perfusion flow rate is necessary. However, the theoretical estimate may give a hint about the range of flow rates appropriate to investigate.

5.9.5 Regulated gene expression by pump switching

By pump switching and thereby changing of cell culture conditions, regulated gene expression of DD-ZsGreen1-DR was demonstrated (Fig. 34), with a good correlation between switching of flow from one pump to another (at addition and removal of Shield1) and expression of DD-ZsGreen1-DR. The time for switching from one liquid to another in the chip has been shown to be between seconds to a few minutes depending on the flow rate [107], which by the expression profile of DD-ZsGreen1-DR is demonstrated to be sufficient to resolve the time scale of the gene expression. By the use of a passive cell culture chip without integrated valves, some leakage from the not flowing inlet cannot be avoided. However, this leakage has by theoretical calculations been estimated to be insignificant [107], and the reversibility of the DD-ZsGreen1-DR gene expression further demonstrated that the leak is too small to influence the biological read-out in this kind of assay.

5.9.6 Limitations of the optimization study of microfluidic perfusion cell culture

The optimization study of the system preparation and the microfluidic cell culture conditions performed in this project is not a complete validation of the developed microfluidic perfusion cell culture system with at least three repetitions of every tested condition/parameter. One of the main objectives of this project was to reach beyond the proof-of-concept level and apply the microfluidic
cell culture system to investigate an issue within stem cell differentiation. However, the results presented in chapter 6 about differentiation of ASCs into adipocytes argue very well that the employed conditions in the microfluidic cell culture system actually were able to support efficient cell function of ASCs in terms of adipogenic differentiation.

5.10 Conclusion and outlook

In this project, we have established procedures to performed successful long-term microfluidic perfusion cell culture of HeLa cells and ASCs with satisfactory cell attachment, morphology and proliferation and without contaminations by bacteria or fungi. In addition, we have applied our developed microfluidic perfusion cell culture system to demonstrate the potential of the system to regulate gene expression of a gene of interest by external actuation and with real-time microscopy detection of the gene expression. Further successful application of the microfluidic perfusion cell culture system to stem cell research by investigation of ASC differentiation into adipocytes is demonstrated in chapter 6.

However, further optimizations have to be done to improve the control of the cell seeding. A cell culture chip design with a closed microfluidic network for cell loading and then cell loading by direct infusion of the cell suspension by a syringe connected to an inlet port could possibly improve the controllability of the cell seeding. Another suggestion could be cell loading through a septum with a syringe and needle. Work is currently going on in the FAST group to improve the cell loading.
Chapter 6

Application to Stem Cell Research

As presented in section 2.1.1 and 2.3.2, cells and in particular stem cells are in a complex microenvironment \textit{in vivo}. In the adult stem cell niches, for example, many interactions and thereby many means of signaling control the fate of the stem cell. It is therefore very difficult to copy this complex microenvironment to \textit{in vitro} cell culture models, in particular, by the use of conventional cell culture methods. As a consequence, the microfluidic cell culture research community has taken this issue, in an otherwise high attention research area, as a great opportunity to provide possibilities for new experimental designs, as reviewed in [27, 28, 123-125]. Gradient generating devices have been applied to study the influence of a particular soluble factor, which may be present in a stem cell niche. For example, activation of the canonical WNT-signaling pathway has been investigated in a WNT3A gradient [126], and differentiation of human neuronal stem cells has been studied by exposing the cells to a gradient of growth factors [29]. Quake \textit{et al.} [5] applied a high-throughput system with integrated automated mixers, peristaltic pumps, pneumatic valves and 96 individually controllable cell culture chambers to investigate the effect of transient stimulation schedules on proliferation and osteogenic differentiation of MSCs. Furthermore, a co-culture device with an array of cell capture cups enabled the study of interactions between up to 6,000 cell pairs of two different cell types [127]. Regarding 3D cultures, the culture of bone marrow MSCs in a 3D environment has been performed by cell seeding in an array of micropillars capturing the cells followed by formation of collagen matrices surrounding the cells [7]. In this way 3D cell-to-cell and cell-to-ECM contacts were enabled. Despite these above-mentioned attempts to more closely
mimic the in vivo stem cell microenvironment, it is still a great challenge to copy the in vivo stem cell niche microenvironment and, in addition, to perform the experiments in a consistent way from one experiment to another [125]. Furthermore, stem cell experiments often require long term cell culture for differentiation to occur, putting further demands on the device performance and experimental optimization.

Besides the above-mentioned possibilities, a general advantage of microfluidic cell culture in relation to stem cell studies is the small cell culture chamber size, which makes it possible to test many conditions by the use of a small number of cells. When dealing with primary cell cultures and maybe slowly dividing cells, the number of cells required for an experiment is important.

### 6.1 Motivation of application

Within the framework of the ProCell project, one of the objectives regarding evaluation was to test the developed device for investigation and optimization of stem cell differentiation in collaboration with the external academic partner, Professor Philippe Collas, University of Oslo. Professor Philippe Collas and his group are, among other things, investigating the differentiation capacity of MSCs from adipose tissue (ASCs), and, in particular, a possible epigenetic programming of MSCs for specific lineages.

As mentioned in section 2.3.4, ASCs have potential as replacement cells in regenerative medicine. In relation to this application, it is crucial to be able to control the differentiation into adipocytes. Furthermore, the differentiation process of ASCs into adipocytes has great interest in basic research. The in vitro differentiation process takes up to two to three weeks. More knowledge about the differentiation process would maybe enable an optimization of the process, and thereby speed up the differentiation and also the research within the differentiation process. Finally, the differentiation of ASCs into adipocytes has an interest in relation to treatment of obesity. More knowledge about the process could provide possible targets in the combating of obesity.

The focus in the ProCell project and the FAST group has been on developing a library of components that enables the creation of fully integrated microfluidic cell culture systems (section 4.2), while less focus has been on developing complex cell culture chips providing possibilities for 3D culture or co-culture experiments. When a system component library has been established as a basis for many possible microfluidic cell culture systems, the focus can then move (and is currently moving) to the cell culture chip level. Thus, at the system developmental stage during this project, we were able to run parallel microfluidic cell cultures in a 2D monolayer with a different number
of cell culture chambers and number of inputs, see section 4.3.6. As described in chapter 5, the systems employed have been optimized to run long-term cell culture experiments without interruptions caused, for example, by bacteria contaminations or introduction of bubbles into the system.

Depending on the applied perfusion flow rate, one of the disadvantages of microfluidic perfusion cell culture is that the constant flow of cell culture medium probably would remove cell secreted signaling factors, as described in section 2.2.1 and 2.2.2. However, this disadvantage can be turned into an advantage. By means of the applied flow rate, the convection in the flow direction can be controlled in relation to the diffusion and reaction time of a signaling molecule. This allows the study of the cellular response at conditions that disrupt a possible paracrine/autocrine signaling. Thus, a microfluidic perfusion culture system can be used to investigate paracrine/autocrine cell signaling in a cell population, even though the signaling factor is not known. Previously, microfluidic perfusion cell culture has been used to reveal paracrine/autocrine signaling involved in the survival of mouse embryonic stem cells (mESCs) [34], and most recently, the use of a microfluidic perfusion cell culture device showed that paracrine/autocrine signaling is involved in both proliferation and neuroectodermal commitment of mESCs [35]. Although a condition of reduced paracrine/autocrine signaling probably can be obtained by conventional cell culture methods by applying low cell density cultures and frequent changes of culture medium, the signaling can probably not be completely controlled, and therefore it may be difficult to obtain a condition with no background signaling.

Paracrine/autocrine signaling has not been directly described to be involved in the differentiation process of a population of MSCs into adipocytes [61, 63, 64, 72, 84, 128, 129]; however, this type of signaling is involved in many cellular processes including processes in developmental biology [48]. Furthermore, high cell confluence (80-90%) has been reported to be required before induction of terminal differentiation by adipogenic stimuli [58, 65], as well as high cell confluence has been shown to promote adipogenesis over osteogenesis of MSCs [66]. Accordingly, it is obvious to speculate that a paracrine/autocrine signaling is involved in the differentiation process of a population of ASCs into adipocytes.

Altogether, this prompted us to apply the developed microfluidic cell culture systems to investigate if paracrine/autocrine signaling actually is involved in the differentiation process of a population of ASCs into adipocytes by examining the effect of different flow rates and compositions of the adipogenic medium on the differentiation process at various cell densities.
6.2 General experimental design

General aspects especially important for the stem cell experiments and the interpretation of the results are described in this section. For more details, see Materials and Methods, Chapter 3. The microfluidic cell culture system 1 (Fig. 10b) was the first system developed during this project and has therefore been applied to most of the stem cell experiments. In all experiments performed with system 1, a gradient of cell density was present along the length axis of the cell culture chamber with the highest cell density at the inlet end of the cell loading (Fig. 35).

![Cell density gradient in relation to flow direction in experiments using system 1. a) Flow direction from the low cell density end to the high cell density end (forward flow). b) Flow direction from the high cell density end to the low cell density end (backward flow).](image)

The gradient of cell density was present due to the fact that the highest possible flow rate was not fast enough to avoid sedimentation of the cells before the entire chamber was loaded with cells (see section 5.5.1). In the first experiments performed, the flow direction was from the low cell density end to the high cell density end (Fig. 35a), with the peristaltic micropump upstream of the cell culture chamber (forward flow) (Fig. 12a). This configuration allowed switching between different pumps and thereby temporal differential cell culture conditions without a manual change of medium in the inlet reservoirs. In addition, this configuration enabled the use of a 24 chamber cell culture chip (Fig. 16d). However, due to problems with precipitations of the serum in the cell culture medium caused by the mechanical influence of the micropump (see section 4.3.1), the configuration was changed to flow from the high cell density end to the low cell density end (backward flow) (Fig. 35b) with the micropump downstream of the cell culture chamber (Fig. 12b). This configuration allowed only the use of an eight chamber cell culture chip (Fig. 16c) connected to one, two or three micropumps depending on the number of applied flow rates in one experiment Fig. 36.

![Different pump connection configurations by the use of an eight chamber cell culture chip. a) One pump connection. b) Two pump connection. c) Three pump connection.](image)
The microfluidic cell culture system 2 (Fig. 10c) enabled uniform cell loading over the entire cell culture chamber (see section 5.5.1), which eliminated the influence of different cell densities over the length axis of the cell culture chamber on the differentiation process/efficiency.

The employed adipose-derived stem cells (ASCs) have been isolated (see section 2.3.4) and provided by the Professor Philippe Collas Laboratory, University of Oslo. The passage of the cells used in the microfluidic cell culture differentiation experiments has been between 6 to 12. In general, the cells have been harvested and loaded into the microfluidic cell culture chambers as described in section 3.2.2.4, 4.3.5 (Fig. 15), and section 5.5. After an initial attachment phase, the ASCs were induced to differentiate into adipocytes the following day in the different tested differentiation conditions by changing the liquid reservoirs with normal cell culture medium to various differentiation media. During cell culture the whole system was placed in an incubator at 37 °C and 5% CO₂. The differentiation was followed for approximately 21 days by microscopy imaging of the whole cell culture chamber, normally every second day. A differentiation period of 21 days is normally needed for efficient and complete differentiation in static cell culture conditions. Due to the fact that fluorescent reporter cells of transcriptional markers of differentiation are not readily available for human ASCs, the accumulation of lipid in intracellular vacuoles has been used as a marker of adipocyte differentiation. In phase contrast microscopy imaging, these lipid-filled droplets appear as white spheres and can therefore easily be marked by imaging analysis software. As a measure of differentiation, the total area of lipid-filled droplets in the entire cell culture chamber was measured and normalized to the total cell area at initiation of differentiation.

6.3 Results

6.3.1 Effect of perfusion on adipocyte differentiation

To investigate if ASCs were able to differentiate into adipocytes at microfluidic perfusion cell culture conditions, the cells were induced to differentiate in normal adipogenic differentiation medium (AM) (DMEM added NCS 15%, IBMX (isobuthyl-methylxanthine) 0.5 mM, dexamethasone 1 µM, indomethacin 0.2 mM and insulin 10 µg/mL). The cells were cultured in system 1 at backward flow and at a flow rate of 125 nL/min, corresponding to an exchange rate of medium in the cell culture chamber of 1.5/hour. The ASCs differentiated and formed lipid-filled droplets (Fig. 37a) in a similar manner to static cell culture (Fig. 37c). Likewise, the characteristic stress fibre formation in the first phase of the differentiation process was observed in perfusion cell culture as in static cell culture (Fig. 37d and e, respectively). Importantly, no spontaneous differentiation was observed when the cells were perfused in normal growth medium (GM) (Fig. 37b).
Fig. 37. Differentiation of ASCs into adipocytes in normal adipogenic differentiation medium (AM) at perfusion cell culture conditions. ASCs were induced to differentiate at a flow rate of 125 nL/min ~ exchange rate of medium in the cell culture chamber of 1.5/hour. a) Imaging at 7, 12, 16 and 22 days after start of differentiation. The cells were able to differentiate and accumulate fat as shown by the lipid-filled droplets indicated by arrows. b) ASCs cultured at perfusion in normal growth medium (GM) as a negative control. No spontaneous differentiation was observed at day 21. c) Differentiation of ASCs at static cell culture conditions at day 21. d) and e). In perfusion cell culture d) the cells formed stress fibers (indicated by arrows) characteristic of the first phase of differentiation in a similar manner to static cell culture e).
Further examination of the effect of perfusion was performed by inducing differentiation in AM at three different flow rates, 33 nL/min, 125 nL/min and 500 nL/min, corresponding to an exchange rate of the medium in the cell culture chamber of 0.4/hour, 1.5/hour and 6/hour. Although large variations in overall differentiation efficiency were observed between different experiments, the differentiation showed a trend, where the efficiency was higher at 125 nL/min than at 33 nL/min, while the differentiation efficiency was much lower at 500 nL/min (Fig. 38a and b). The large variations in overall differentiation efficiency from one experiment to another were probably caused by varying cell densities at the onset of differentiation. It is well known that the cell density affects the adipogenic differentiation efficiency [58, 65, 66].

**Fig. 38. Effect of perfusion flow rate on the differentiation efficiency of ASCs cultured in AM.** ASCs were induced to differentiate in normal adipogenic medium (AM) at 33 nL/min, 125 nL/min or 500 nL/min, corresponding to an exchange rate of the medium in the cell culture chamber of 0.4/hour, 1.5/hour and 6/hour. 

*a)* Imaging of a representative experiment at day 20 after start of differentiation. 10x phase contrast images.

*b)* The total area of lipid-filled droplets is measured at day 13-14 for three representative examples. The area of lipid-filled droplets is measured by using ImageJ and normalized to the total cell area at the start of differentiation.

Furthermore, the cells grown at a flow rate of 33 nL/min had an almost round cell shape, while more elongated cell morphology was observed at a flow rate of 500 nL/min. The differences in cell morphology and differentiation efficiency between the flow rates of 33 nL/min and 500 nL/min...
could be caused by shear effects of the flow or disruption of a possible paracrine/autocrine signaling involved in adipocyte differentiation, which is further investigated in the next section.

### 6.3.2 Influence of conditioned medium on adipocyte differentiation

In order to investigate whether the low differentiation efficiency at 500 nL/min was caused by shear effects or by interruption of a possible paracrine/autocrine signaling involved in adipocyte differentiation, ASCs were induced to differentiate in either normal adipogenic medium (AM) or a 1:1 mixture of conditioned medium (CM) (medium from ASCs undergoing adipogenic differentiation in static culture) and AM with 1.5 times the concentration of adipogenic stimuli (1.5x AM). If the cells secrete one or more factors involved in adipocyte differentiation, the CM from differentiating cells would contain those cues. The CM was mixed 1:1 with fresh AM to ensure sufficient supply of nutrients. 1.5x AM was applied instead of 1x AM to compensate for an expected consumption/degradation of the adipogenic stimuli in the collected CM. Again the cells were induced to differentiate at the three flow rates 33 nL/min, 125 nL/min and 500 nL/min either by the use of system 1 or system 2. As shown in Fig. 39, the differentiation efficiency was increased when perfused with 1.5x AM+CM compared to AM alone. The enhanced differentiation efficiency was observed for all three flow rates. However, the effect of the conditioned medium was batch dependent. Fig. 39b) and d) show a representative example of CM batches with a high adipogenic effect, while Fig. 39c) and e) show an example of a CM batch with a lower adipogenic effect. Besides variations of CM from one batch to another, the adipogenic effect was only present when applying a freshly collected batch (Fig. 39b, d and Appendix 1), suggesting that the secreted paracrine/autocrine signaling factor(s) are quite unstable. All collected results from 9 independent experiments are shown in Appendix 1. To ensure that the increased differentiation efficiency was not due to the higher concentration of adipogenic stimuli in 1.5x AM+CM, 1.5x AM alone was tested. However, this resulted in lower differentiation efficiency than 1x AM (data not shown). In addition, ASCs grown in GM alone showed no spontaneous differentiation (data not shown). Thus, the results suggest that there exist paracrine/autocrine signaling factors that enhance adipogenic differentiation.

Although, the differentiation efficiency was increased when perfusing with 1.5x AM+CM, the overall efficiency was still lower when perfusing at 500 nL/min compared to 125 nL/min. This lower differentiation efficiency could still be due to shear effects or it could possibly be due to higher exposure of the cells to the adipogenic stimuli supported by the fact that 1.5x AM reduced differentiation efficiency.
Fig. 39. **Effect of conditioned medium on adipocyte differentiation.** ASCs were induced to differentiate at different flow rates, 33 nL/min, 125 nL/min and 500 nL/min in either AM or 1.5x AM+CM (a 1:1 mixture of 1.5 times the concentration of AM and CM collected from differentiating cells in static culture). Differentiation is measured by the total area of lipid-filled droplets using ImageJ and normalized to the total cell area at the start of differentiation. a) Imaging of representative examples at day 20 after start of differentiation. 10x phase contrast images. b) The differentiation efficiency shown at day 18 after onset of differentiation. Fresh batch CM number 2 was used for flow at 33 nL/min, while fresh batch CM number 3 was used for 125 nL/min and 500 nL/min. c) The differentiation efficiency shown at day 20 after onset of differentiation. Not freshly collected CM batch number 2 was used at all three flow rates. d) Differentiation over time shown for the same experiments as in b). e) Differentiation over time shown for the same experiments as in c). All collected results from 9 independent experiments are shown in Appendix 1.
6.3.3 Effect of concentrations of adipogenic stimuli on adipocyte differentiation

To examine whether the lower differentiation efficiency at a flow rate of 500 nL/min was caused by possible shear effects or too high an exposure of the cells to the adipogenic stimuli, ASCs were induced to differentiate at a flow rate of 500 nL/min in AM, 1.5x AM+CM 1:1, AM diluted 4x or 1.5x AM diluted 4x+CM 1:1. Furthermore, to test the influence of cell density, the cells were loaded at different cell suspension densities, 0.5x10^5, 2x10^5 or 4x10^5 cells/mL. All conditions were tested in one experiment by the use of a 16 chamber cell culture chip (Fig. 16a, but with only one inlet to each chamber) and system 2.

As shown in Fig. 40, differentiation in medium with a lower level of the added adipogenic factors, but with a high level of CM factors (1.5x AM diluted 4x+CM 1:1) resulted in a large increase in differentiation efficiency compared to the mixture of AM and CM with a high level of the added adipogenic factors (1.5x AM+CM) (Fig. 40b, c, d and e). As a reference ASCs were differentiated in diluted AM without CM (AM diluted 4x), which resulted in the same differentiation efficiency as in 1.5x AM+CM. Thus, the increased differentiation efficiency observed when differentiating in a medium with a lower level of the added adipogenic factors, but with a high level of CM factors (1.5x AM diluted 4x+CM 1:1) was not due to a dilution of the added adipogenic factors alone. Accordingly, the results further suggest a role of paracrine/autocrine signaling factors in adipocyte differentiation. Preliminary experiments investigating the effect of a dilution of both the added adipogenic factors and the CM factors ((1.5x AM+CM) diluted 4x) showed only a minor positive effect on differentiation compared to not diluted AM+CM (1.5x AM+CM) and diluted AM (AM diluted 4x) (data not shown). This further indicates an existence of cues in CM having an effect on adipocyte differentiation, and that the signaling factors in CM needs a certain threshold concentration to have an effect on adipocyte differentiation. The increased adipogenic effect of 1.5x AM diluted 4x+CM compared to 1.5x AM+CM was approximately 7-fold, 3-fold and 2-fold for the cell loading suspension densities 0.5x10^5, 2x10^5 and 4x10^5 cells/mL, respectively. Compared to AM the increase was even higher, approximately 13-fold, 17-fold and 4-fold for the cell densities 0.5x10^5, 2x10^5 and 4x10^5 cells/mL, respectively. As seen in Fig. 40a) the differentiation efficiency at 1.5x AM diluted 4x+CM at the highest cell density achieved almost complete differentiation of all the cells. Besides the above observations, the overall differentiation efficiency rose in relation to increasing cell density (Fig. 40a and b), further indicating a positive influence from signaling factors secreted by the cells during the course run of the experiment. However, an almost zero-background differentiation was observed for AM, 1.5x AM+CM and AM diluted 4x at a cell loading suspension density of 0.5x10^5 cells/mL and for AM at a cell loading suspension density of 2x10^5 cells/mL.
Fig. 40. Effect of concentrations of adipogenic stimuli on adipocyte differentiation. ASCs were loaded at different cell suspension densities, 0.5x10^5, 2x10^5 or 4x10^5 cells/mL, and induced to differentiate at a flow rate of 500 nL/min in AM, 1.5x AM+CM 1:1, AM diluted 4x or 1.5x AM diluted 4x+CM 1:1. All conditions were tested in one experiment by the use of a 16 chamber cell culture chip. Differentiation is measured by the total area of lipid-filled droplets using ImageJ and normalized to the total cell area at the start of differentiation. The results shown are from one experiment out of two independent experiments. Results from the other experiment are shown in Appendix 2. a) Imaging at day 16 after start of differentiation. 10x phase contrast images. b) The differentiation efficiency shown at day 18 after onset of differentiation. c), d) and e) The differentiation shown over time for the cell loading suspension densities 0.5x10^5, 2x10^5 and 4x10^5 cells/mL, respectively.
Altogether, the results add more evidence to the suggestion of an existence of paracrine/autocrine signaling factors involved in adipocyte differentiation.

Interestingly, AM diluted 4x resulted in an increased differentiation efficiency compared to AM, suggesting a negative effect on differentiation caused by too high an exposure of the cells to the adipogenic stimuli and not caused by shear stress effects.

The results shown in Fig. 40 are from one experiment out of two independent experiments. The results from the other experiment are shown in Appendix 2. Again a large variation in overall differentiation efficiency was observed, however the trend with a great positive effect of 1.5x AM diluted 4x+CM on differentiation was similar. The variation in overall differentiation efficiency between the two experiments was probably again due to differences in cell densities at onset of differentiation and CM batch variations employed in the two experiments.

6.3.4 Static cell culture reference

As a reference to the shown effect of CM and concentrations of adipogenic stimuli on adipocyte differentiation in perfusion culture, ASCs were seeded at a cell density of 40, 160 or 640 cells/mm$^2$ in a T25 cell culture flask and induced to differentiate in AM, 1.5x AM+CM, AM diluted 4x or 1.5x AM diluted 4x+CM at static cell culture conditions. The entire medium was changed every 4 days.

**Fig. 41. Effect of conditioned medium and concentrations of adipogenic stimuli on adipocyte differentiation in static cell culture.** ASCs were seeded at 40, 160 or 640 cells/mm$^2$ in a T25 cell culture flask and induced to differentiate in AM, 1.5x AM+CM, AM diluted 4x or 1.5x AM diluted 4x+CM at static cell culture conditions. All medium was changed every 4 days. a) Imaging at day 16 after start of differentiation. 10x phase contrast images. b) The differentiation efficiency shown at day 20 after onset of differentiation. Differentiation is measured by the average of the total area of lipid-filled droplets of 4 areas of 7.7 mm$^2$ each. The area of lipid-filled droplets is measured by using ImageJ and normalized to the total cell area at the start of differentiation.
Overall, the differentiation efficiency as in perfusion culture was observed to be cell density dependent with increasing differentiation at higher cell densities (Fig. 41a and b). Furthermore, both 1.5x AM+CM and 1.5x AM diluted 4x+CM resulted in an increased differentiation efficiency compared to AM (Fig. 41a and b). For 1.5x AM+CM over AM, a 2-fold, 3-fold and 1.4-fold increase was observed at cell densities of 40, 160 and 640 cells/mm², respectively. A slightly higher increase was seen for 1.5x AM diluted 4x+CM over AM with a 3.5-fold, 3.5-fold and 2-fold increase at cell densities of 40, 160 and 640 cells/mm², respectively. However, the rise in differentiation efficiency caused by addition of CM was much lower compared to perfusion cell culture (Fig. 41 and Fig. 40). At the high cell densities, the lower increase of differentiation was due to a larger differentiation efficiency in AM in static culture compared to perfusion culture (Fig. 41 and Fig. 40). This indicates a greater influence of signaling factors secreted by the cells during the experiment in static cell culture compared to perfusion culture. Thus, the results in static cell culture conditions supported the results observed in perfusion cell culture conditions.

A minor positive effect of AM diluted 4x on the differentiation efficiency compared to AM was observed (Fig. 41b). Combined with a slightly higher differentiation efficiency in 1.5xAM diluted 4x+CM compared to 1.5x AM+CM, these results indicate that a lower concentration of the adipogenic stimuli also increases the differentiation efficiency in static cell culture. However, the difference in differentiation efficiency between AM diluted 4x and normal AM was much lower in static cell culture conditions compared to perfusion culture conditions (Fig. 41 and Fig. 40).

6.3.5 Spatial differences in adipocyte differentiation in relation to flow

To investigate the spatial adipocyte differentiation efficiency along a gradient of cell densities in the cell culture chamber, ASCs were induced to differentiate at different flow directions (forward or backward flow) in relation to the cell density gradient obtained by the use of system 1 (see section 6.2 and Fig. 35). The cells were induced to differentiate at different flow rates (33 nL/min, 125 nL/min or 500 nL/min) in either AM or 1.5x AM+CM. Profiles of differentiation along the cell culture chamber were created by measuring the sum of white pixels (lipid pixels) across the cell culture chamber and along the length axis of the cell culture chamber (x-axis) at different time points after onset of differentiation (y-axis) (Fig. 42). The color code indicates the differentiation efficiency with the highest differentiation efficiency at the brightest areas.

At a flow rate of 33 nL/min and a flow direction from the low cell density area to the high cell density area (forward flow) (Fig. 42a), a higher differentiation was seen in the high cell density end of the chamber compared to the low cell density end. The spatial analysis of differentiation at for-
ward flow direction has only been analyzed at a flow rate of 33 nL/min due to problems with precipitations from the serum in the cell culture medium at the high flow rates (see section 4.3.1).

Fig. 42. Spatial effect of flow on adipocyte differentiation. ASCs were induced to differentiate at different flow directions (forward or backward flow) in relation to the cell density gradient and at different flow rates (33 nL/min, 125 nL/min or 500 nL/min) in either AM or 1.5x AM+CM. Profiles of differentiation along the cell culture chamber were created by measuring the sum of white pixels (lipid pixels) across the cell culture chamber and along the length axis of the cell culture chamber (the x-axis) at different time points (days) after onset of differentiation (the y-axis) by ImageJ. The color code indicates the differentiation efficiency by the number of white pixels (lipid-pixels). a) Spatial differentiation efficiency at a flow rate of 33 nL/min and flow direction in relation to cell density gradient as in Fig. 35a. b) Spatial differentiation efficiency at a flow rate of 33 nL/min and flow direction in relation to cell density gradient as in Fig. 35b. c) Spatial differentiation efficiency at a flow rate of 125 nL/min and flow direction in relation to cell density gradient as in Fig. 35b. d) Spatial differentiation efficiency at a flow rate of 500 nL/min and flow direction in relation to cell density gradient as in Fig. 35b. Plots were made in MatLab by Søren Vedel.
When changing to a flow direction from the high cell density area to the low cell density area (backward flow), a similar degree of differentiation was observed in the low cell density area compared to the high cell density area at flow rates of 33 and 125 nL/min (Fig. 42b and c). The higher differentiation efficiency in the low cell density areas at flow direction from the high cell density area to the low cell density area may be caused by a flow of cell secreted signaling factors from the cells in the high cell density area, which thereby can interact with the cells in the low cell density area.

As observed previously (Fig. 38, Fig. 39, Fig. 40), a very low differentiation efficiency was seen in AM at a flow rate of 500 nL/min (Fig. 42d), which indicates that the flow rate of 500 nL/min removes most of the cell secreted signaling molecules, and thereby interrupts paracrine/autocrine cell-to-cell signaling. The areas, indicating differentiation already from day 0 is not an indication of differentiation (Fig. 42d), but an artifact caused by particles in the cell culture chamber.

An effect of CM on adipocyte differentiation was observed at all three tested flow rates (33, 125 and 500 nL/min). However, the highest increase in differentiation efficiency caused by CM was seen at a flow rate of 500 nL/min (Fig. 42d) and in the low cell density end at forward flow at a flow rate of 33 nL/min (Fig. 42a). Thus, as shown in Fig. 40 the highest effect of CM is observed at the lower cell density cultures.

### 6.3.6 Dependence of nutrient supply on adipocyte differentiation

The fat accumulation in the form of formation of intracellular lipid-filled droplets is the final step in adipocyte differentiation. This is a metabolic process that probably requires a large supply of nutrients compared to requirements during normal cell maintenance. To investigate the effect of nutrient availability on the adipocyte differentiation in terms of fat accumulation, ASCs were induced to differentiate in 1.5x AM+CM at a flow rate of 33 nL/min or 500 nL/min. Because high cell density cultures are expected to require a larger nutrient supply than low cell density cultures, the fat accumulation was further examined at three different cell loading suspension densities, 0.5x10^5, 2x10^5 and 4x10^5 cells/mL.

The two lowest cell densities (0.5x10^5 and 2x10^5 cells/mL) resulted in almost the same amount of accumulated fat at both flow rates (Fig. 43b and c). In contrast, a large difference was observed at the highest cell loading suspension density (4x10^5 cell/mL).
Fig. 43. Dependence of nutrient supply on adipocyte differentiation. ASCs were induced to differentiate in 1.5x AM+CM at a flow rate of 33 nL/min or 500 nL/min. Furthermore, the differentiation was analyzed at three different cell loading suspension densities, 0.5x10^5, 2x10^5 or 4x10^5 cells/mL. Differentiation is measured by the total area of lipid-filled droplets using ImageJ. a) Formation of lipid-filled droplets in the high cell density culture (4x10^5 cells/mL) at day 5 and day 20 at a flow rate of 33 nL/min and 500 nL/min. b) Differentiation shown over time at a flow rate of 33 nL/min. c) Differentiation shown over time at a flow rate of 500 nL/min. One experiment has been performed at each flow rate.
At a flow rate of 33 nL/min, the fat accumulation in the high cell density culture rose quickly above the level observed for the low cell density cultures during the first 2-8 days after onset of differentiation (Fig. 43a and b), with a high percentage of the cells having accumulated a few lipid-filled vacuoles at day 5 (Fig. 43a). This indicates a possible influence of cell secreted signaling factors on adipocyte differentiation in the high cell density cultures at low flow rates.

However, in contrast to the slope of the curve for the medium cell density culture (2x10^5 cell/mL), the slope of the curve for the high cell density culture was almost constant during the whole experiment (Fig. 43b), and in fact the total fat accumulation was lower in the high cell density culture (4x10^5 cell/mL) compared to the medium cell density culture (2x10^5 cell/mL) at a flow rate of 33 nL/min at day 20(Fig. 43b). This indicates too low a supply of nutrients for the fat metabolism to occur in the high cell density culture at 33 nL/min. Conversely, the slope of fat accumulation for the highest cell density cultures (4x10^5 cells/mL) was much higher when the cells were induced to differentiate at a flow rate of 500 nL/min (Fig. 43c) and the total fat accumulation in the high cell density culture was 4-fold higher at a flow rate of 500 nL/min compared to a flow rate of 33 nL/min, shown by more cells with lipid-filled droplets, more lipid-filled droplets in each cell and a bigger size of the lipid-filled droplets (Fig. 43a and c). One experiment has been performed for each flow rate.

Thus, as expected the supply of nutrients seemed to have an effect on the fat accumulation efficiency and thereby the adipocyte differentiation as measured by the amount of lipid accumulation in the cells. At high cell density cultures, a flow rate of 500 nL/min could sustain a much higher fat accumulation rate than 33 nL/min.

6.4 Discussion

In this application of the developed microfluidic cell culture systems, we have investigated whether paracrine/autocrine signaling is involved in the differentiation of a population of ASCs into adipocytes and thereby, also, whether microfluidic cell culture systems can be used as a tool to investigate paracrine/autocrine signaling within a cell population. We took advantage of the inherent property of constant perfusion cell culture, in which, depending on the applied flow rate, secreted signaling molecules are washed away, and thereby probably disrupt a possible paracrine/autocrine signaling.
6.4.1 Main findings

6.4.1.1 Paracrine/autocrine signaling is involved in adipocyte differentiation

When ASCs were induced to differentiate in AM, a flow rate of 500 nL/min resulted in a lower differentiation efficiency compared to flow rates of 33 nL/min and 125 nL/min, which indicated an interruption of a possible paracrine/autocrine signaling implicated in the differentiation process (Fig. 38). The differentiation efficiency was rescued by differentiation in a 1:1 mixture of AM and CM (collected from differentiating cells in a static cell culture), and, in fact, an increase in differentiation efficiency was observed at all three flow rates when perfusing with CM (Fig. 39). This positive effect on the differentiation by CM suggests an involvement of paracrine/autocrine signaling in adipocyte differentiation. Further evidence supporting the participation of paracrine/autocrine signaling in adipocyte differentiation is demonstrated by the observation that a dilution of the normal adipogenic factors in the AM+CM mixture increased even further the positive effect of CM (Fig. 40). In addition, this increased positive effect was not observed when the cells were induced to differentiate in diluted AM alone (Fig. 40) or in a dilution of both AM and CM (data not shown). The increased differentiation efficiency observed by differentiation in CM was supported by data from static cell culture conditions, although the difference in differentiation efficiency was lower than that observed in perfusion cell culture (Fig. 41b).

Besides the positive influence of CM on differentiation, a general increase in differentiation efficiency was seen at increasing cell densities, which previously has been described in the literature to promote adipogenesis [58, 65, 66]. McBeath et al. [66] explained that the high cell densities resulted in cells with a round cell shape, and that the round cell shape was the trigger for adipogenesis. However after exposure of ASCs to AM, the first sign of differentiation is actually a change in cell shape from an elongated, spindle-like shape to a more round or oval cell shape, indicating that the round cell shape is not a trigger of differentiation, but a first step in the differentiation process. Another explanation of the large differentiation efficiency in high cell density cultures, which is supported by the data shown in this study, could be that the high cell density results in a threshold concentration of paracrine/autocrine signaling molecules, and that this threshold concentration can be sensed by the nearby cells due to the short distances between the cells in a dense culture.

Although adipocyte differentiation has been reviewed/discussed in several publications [61, 63, 64, 72, 84, 128, 129], paracrine/autocrine signaling within a ASC population has to our knowledge not been described to be involved in the differentiation process of ASCs into adipocytes. However, in a study by Maxson et al. [130], the effect of conditioned media on adipocyte and osteoblast dif-
Differentiation has been investigated in static cell cultures by the use of conventional cell culture methods (i.e. in petri dishes). A 1:1 mixture of AM and CM collected from osteoblast differentiation was shown to enhance adipogenic differentiation by a factor of 2.7, which is similar to the increase effect of CM on adipogenic differentiation that we observed in static cell cultures (Fig. 41). Likewise, a 1:1 mixture of osteogenic medium and CM collected from adipocyte differentiation was shown to enhance osteogenic differentiation by a factor of 1.4. However, by the use of a microfluidic perfusion cell culture system we demonstrated an increased adipogenic effect of CM compared to AM of 17-fold (Fig. 40). Later Maxson et al. have shown the same phenomenon between chondrocyte CM and osteoblast CM [131]. These results, together with the results demonstrated in this thesis, indicate that there might exist a common paracrine/autocrine factor influencing/enhancing the differentiation of MSCs towards the mesodermal lineages.

Based on the knowledge described in the literature and the data presented here, a simplified model of exogenous factors influencing adipocyte differentiation can be created, as shown in Fig. 44. The model shows that an effective concentration of adipogenic stimuli induces adipocyte differentiation, which is then further affected/enhanced by paracrine/autocrine signaling. The effect of the paracrine/autocrine signaling on the fat metabolism and accumulation may be direct or indirect through commitment of differentiation or through activation of the transcriptional cascade in the terminal differentiation process. Furthermore, fat metabolism may be dependent on nutrient availability, as well as the first steps in adipocyte differentiation are possibly dependent on a certain nutrient level.

**Fig. 44. Simplified model of exogenous factors affecting adipocyte differentiation.** An effective concentration of adipogenic stimuli induces adipocyte differentiation, which is affected/enhanced by paracrine/autocrine signaling. The effect of paracrine/autocrine signaling on fat metabolism may be direct or indirect through commitment of differentiation or through activation of the transcriptional cascade in the terminal differentiation process. The fat metabolism may furthermore be dependent on nutrient availability, as well as the first steps in adipocyte differentiation possibly are dependent on a certain nutrient availability.
6.4.1.2 Optimal concentrations of cues may differ from static culture to perfusion culture

As shown in Fig. 38, a lower differentiation efficiency was observed at the highest flow rate. As maybe expected, this was not caused by shear effects of the flow, but demonstrated to be caused by high exposure of the cells to the adipogenic stimuli. A 4 times dilution of the added adipogenic stimuli increased the differentiation efficiency both in AM alone and in the mixture of AM and CM. Actually, a previous study [130] also showed an enhancement of adipogenic differentiation when differentiation was induced in a 1:1 mixture of AM and osteogenic medium (i.e. a 2x dilution of the added adipogenic factors). At cell culture under static conditions, the concentration of adipogenic stimuli is expected to decrease during the period between changes of medium caused by consumption and degradation of the adipogenic molecules. However, in perfusion cell culture the medium is constantly renewed resulting in a higher total exposure of the cells to the adipogenic cues. The used concentrations of the added adipogenic stimuli have been optimized at static cell culture conditions and may therefore not be optimal for adipocyte differentiation in perfusion cell culture. Thus, the results indicate that concentrations of various factors, in general, possibly cannot be transferred to perfusion cell culture without some preliminary validation. Issues related to microfluidic cell culture have been described in several reviews [1, 13, 47, 132]. However, when describing mass transport the concern is about cell viability/proliferation in relation to flow rate and thereby supply of growth factors and nutrients. None of them discuss the concentrations of exogenous stimuli in microfluidic perfusion cell cultures vs. macro scale cultures. A too high exposure of cells to an exogenous factor may have a negative impact on cell viability, which could be misinterpreted as a negative effect caused by other system properties.

6.4.1.3 Supply of nutrients may affect terminal adipocyte differentiation

Accumulation of fat in intracellular vacuoles has been used as a marker of adipocyte differentiation in this study. Besides the signaling cascades involved in induction of adipocyte commitment and terminal differentiation, the fat accumulation involves metabolic processes, which probably are dependent on sufficient nutrient supply. Thus, when investigating the involvement of paracrine/autocrine signaling in the adipocyte differentiation process with the use of fat accumulation as a marker of differentiation, it is very important that the fat accumulation is not inhibited by nutrient depletion. At a low flow rate of 33 nL/min differentiation of a high cell density culture resulted in less fat accumulation compared to a lower cell density culture (Fig. 43b), whereas the fat accumulation in a high cell density culture at a high flow rate of 500 nL/min was much higher compared to a lower cell density culture (Fig. 43c). Thus, in the microfluidic cell culture systems employed here, a flow rate of 500 nL/min can sustain a much higher fat accumulation rate than 33 nL/min in the high cell density cultures.
However, in a constant perfusion cell culture the available nutrients are not linear proportional to the applied flow rate, due to the fact that the time for a molecule to diffuse across the chamber down to the cells may be slower than the flow of the molecule in the perfusion flow direction. For example, in a chamber with a height of 0.5 mm (as in the cell culture chips employed here), it takes a sugar molecule with a diffusion coefficient of $5 \times 10^{-10} \text{ m}^2/\text{s}$ [133] 250 sec to diffuse from the top of the chamber to the bottom. At a flow rate of 500 nL/min (~ velocity of $1.1 \times 10^{-5} \text{ m/s}$), it takes the sugar molecule only 45 sec to move 0.5 mm in the perfusion flow direction, implying that the sugar molecule moves much faster in the flow direction than by diffusion across the chamber. Thus, at a flow rate of 500 nL/min the cells can only take advantage of the nutrients in the lower layer of the cell culture medium. Altogether, the results indicate that a flow rate sufficient for normal cell maintenance and proliferation is maybe not sufficient for high nutrient demanding metabolic processes as extensive fat synthesis to occur. Work is currently going on in the ProCell project by the theoretical microfluidics partners to develop a model of fat accumulation in relation to applied flow rates, diffusible signaling and supply of nutrients. The effect of nutrient availability on fat accumulation in relation to adipocyte differentiation can be further examined by investigating differentiation in media with high or low glucose content.

6.4.1.4 Microfluidic perfusion cell culture is a useful tool in investigation of paracrine and/or autocrine cell signaling

To investigate paracrine/autocrine signaling within a population of cells, it is important to be able to create a low background signaling level to reveal a possible effect of added paracrine/autocrine factors, for example in the form of a conditioned medium. By the use of the developed microfluidic systems we demonstrated up to a 13-17 fold increase in differentiation efficiency at the two lowest tested cell densities by perfusion with CM compared to perfusion with AM alone (Fig. 40b, c and d). Thus, it was shown that we could remove cell secreted signaling molecules to an extent that resulted in a biological read-out (low differentiation), and that these signaling molecules were reintroduced by perfusion with CM. The increased effect of CM compared to AM alone was observed at the same cell density and flow rate and was therefore not due to any system effects (Fig. 40). In another recent study [35] using microfluidic perfusion culture device at similar flow conditions (see below) to investigate paracrine/autocrine signaling involved in neuroectodermal differentiation of mESCs, a 5.6-fold increase in Sox-1 (a marker of differentiation) expressing cells was observed at differentiation in CM compared to differentiation in medium without CM. However, by the use of microfluidic perfusion cell culture we demonstrated a 17-fold increase in adipocyte differentiation by perfusion with CM compared to perfusion with AM (Fig. 40). Ellison et al. [34] have used a microfluidic perfusion cell culture device to investigate cell
viability of mESCs at conditions, were the medium in the cell culture chamber was washed away at different defined time intervals (every 0 minutes (constant perfusion) to 720 minutes). They observed a decrease of 50% in cell viability at 0 minutes intervals compared to 720 minutes intervals. However, they did not examined cell viability in CM.

The corresponding results obtained in this project in static cell culture were an increase in differentiation efficiency of only 3.5-fold (Fig. 41b), which is similar to what was observed in a study of the effect of osteoblast CM on adipocyte differentiation in a static cell culture by Maxson et al. [130]. They observed a 2.7-fold increase in cellular lipid content compared to cells not exposed to CM during differentiation. The lower difference in differentiation efficiency between differentiation in CM and AM alone in static cell cultures suggests that the concentration of cell secreted cues between changes of medium increases to a level that results in a higher background signaling than in perfusion cell culture and thereby a higher differentiation efficiency at differentiation in AM alone.

However, a lower difference between CM and AM was observed at the highest cell densities in both perfusion and static cell culture conditions, with a 4-fold and 2-fold increase, respectively (Fig. 40a and e, and Fig. 41b). The small differences suggest that the assay at the high cell densities was “contaminated” by signaling molecules secreted by the cells during the experiment, which therefore resulted in a higher background signaling. It should be noted that the actual cell density at onset of differentiation at the high cell density in perfusion culture was 900 cells/mm², while the corresponding actual cell density in static culture was 640 cells/mm². Thus, the results are therefore not fully comparable.

The higher background differentiation in perfusion cell culture at the high cell density (Fig. 40b and e), indicates that a higher flow rate is needed to remove the background signaling during the run of the experiment. The relation between the axial convection time and transverse diffusion time can be estimated by the Péclet number given by $Pe = v \cdot l / D$, where $v$ is the fluid velocity, $l$ is the characteristic length of the system (here the height of the cell culture chamber) and $D$ is diffusion coefficient of a given molecule. As an example of a possible signaling molecule, Dickkopf-1 (MV 28.672 Da), which has been associated with adipocyte differentiation, is used. Dickkopf-1 is a cell secreted molecule that has been shown to inhibit WNT signaling [76], which is involved in negative regulation of adipocyte differentiation. The diffusion coefficient of an unknown protein can be estimated by equation (5) (section 2.2.2) and with the viscosity of the medium set to the viscosity of water (1.002 mPa·s), the diffusion coefficient of Dickkopf-1 (MV 28.7 Da) is calculated to $7.97 \times 10^{-11}$ m²/s. With a flow rate of 500 nL/min (~ velocity of $1.1 \times 10^{-5}$ m/s) and a cell culture
chamber height of 0.5 mm, the Pécle number is calculated to 69, which is indicative of a mass transport dominated by convection. In the study by Blagovic et al. [35] using microfluidic perfusion culture to investigate paracrine/autocrine signaling involved in neuroectodermal differentiation of mESCs, flow conditions with a calculated Pécle number of 75 was shown to reduce diffusible signaling to an extend that negatively affected neuroectodermal differentiation of mESCs. But although the Pécle number at our flow conditions is indicative of convection dominated mass transport, the diffusion times at the very short distances between the cells down at the cell layer in the cell culture chamber is much faster, which may be the cause of the observed background differentiation at high cell densities. As mentioned in section 6.4.1.3, work is currently going on in the ProCell project by the theoretical microfluidics partners to develop a model of fat accumulation in relation to applied flow rates, diffusible signaling and supply of nutrients.

In addition, the spatial analysis of the effect of changing flow direction on differentiation over a gradient of cell densities showed that the microfluidic perfusion cell culture system is useful for investigations of cell-to-cell communications between for example high cell density areas and low cell density areas.

Altogether, the results show that the employed microfluidic perfusion cell culture system is a more powerful tool than conventional static cell cultures in investigations of paracrine/autocrine signaling within a cell population.

Another way to investigate paracrine/autocrine signaling by the use of a microfluidic cell culture system could be by a recirculation of the cell culture medium and with no recirculation in the references. However, this makes the system design more complex.

6.4.2 Encountered problems and limitations of the study

6.4.2.1 Variations in differentiation efficiency

Large variations in differentiation efficiencies were observed from one experiment to another. These variations were primarily caused by the fact that it has not been possible to control the actual seeding density in the cell culture chambers with the current design/configuration of the microfluidic cell culture system (see section 5.5.2). Previous studies [58, 65, 66] and data shown in this project (Fig. 40 and Fig. 41) have demonstrated that the cell density in particular has a great influence on the adipogenic differentiation efficiency. Thus, for further studies the cell loading method needs to be optimized to be able to control the actual cell seeding density. To avoid sedimentation of the cells during loading, a direct injection method may be better. However, our experiences have shown that it is not an easy task to perform the cell loading by direct injection without introducing
Chapter 6

bubbles into the microfluidic system. As mentioned in section 4.6.5, solutions for cell loading presented in the literature are normally infusion/withdrawal from various, mostly not integrated, cell reservoirs by manual use of a syringe [32, 35, 36, 113, 115, 116], or by peristaltic pumping / differential pressure application [5, 7]. These methods often employ a separate microfluidic network and integrated valves for the cell loading process, whereby introduction of bubbles into the whole microfluidic system is avoided [35]. Work is currently going on in the FAST group to improve the cell loading.

Another reason for some of the variations in differentiation efficiencies is that the method for normalization of the area of lipid-filled droplets is not optimal. Due to the known influence of cell density on differentiation efficiency and general variations in cell number, it has been necessary to normalize the area of lipid-filled droplets in relation to the cell density. The most optimal method would have been to normalize the area of the lipid-filled droplets to the number of cells. However, because of a very low contrast of the ASCs in microscopy imaging and a very spread/spindle-like morphology, it has not been possible to perform the cell counting by image analysis software (even by specialists) and a manual counting of the cells in all experiments would have been very time-consuming. Accordingly, the cell area was chosen for normalization, although the area is not always proportional to the number of cells, in particular at high cell densities, where the area of each cell is decreased because of limited space. Cells genetically modified with a fluorescent reporter to a nucleus protein would solve the cell counting problem, but fluorescent reporter cells are not readily available for human ASCs.

A final reason is batch variations of the collected CM. The concentration of cell secreted cues in CM is probably dependent on the cell density and thereby also the differentiation efficiency in the cultures from which the CM is collected. Furthermore, our experiences showed that a freshly collected batch had a higher adipogenic effect than an older batch, thereby indicating that the paracrine/autocrine signaling molecules are quite unstable. The CM was aliquoted and stored at -20 °C until use. Furthermore, CM stored at 4 °C has been tested, but showed the same effect on differentiation as CM stored at -20 °C (data not shown).

Although the large observed variations in differentiation efficiencies, the trend when using a freshly collected batch of CM was an enhanced effect of CM on adipocyte differentiation compared to differentiation in AM alone.
6.4.2.2 Markers of adipocyte differentiation

In this project, the formation of lipid-filled droplets has been used as a marker of adipocyte differentiation. At phase contrast microscopy imaging, these lipid-filled droplets appear as white spheres and can therefore easily be marked by imaging analysis software. However, this is a very late marker of differentiation. Furthermore, as described in section 6.3.6 and 6.4.1.3, the fat metabolism is dependent on sufficient nutrient supply, which potentially can interfere with the investigation of the signaling process involved in the adipocyte differentiation and thereby lead to misinterpretations. Despite these disadvantages, the formation of lipid-filled droplets was chosen as a first choice for several reasons. In general, the possible read-out methods in relation to microfluidic cell culture are more limited than in conventional cell culture studies, mainly due to the fact that the small sample size is often incompatible with conventional read-out methods, such as Western immunoblotting, flow cytometry and reverse-transcription PCR (RT-PCR). Furthermore, we wanted to make a time-lapse study of the differentiation process and not just an end-point study. In addition to fat accumulation as a marker of differentiation, the optimal solution for a time-lapse study of the differentiation process would have been the use of fluorescent reporter cells of some of the early markers of the adipogenic differentiation process, such as the transcription factors C/EBPβ, C/EBPδ, PPARγ2 and C/EBPα. However, as mentioned before fluorescent reporter cells are not readily available for human ASCs. The use of more markers of the adipogenic differentiation would add more evidence to the question of the role of paracrine/autocrine signaling in adipocyte differentiation. And most importantly, by the use of some of the early transcriptional markers, it can be further investigated where a paracrine/autocrine signaling act in the process of adipocyte differentiation.

6.5 Conclusion and outlook

By the use of our developed microfluidic cell culture systems, we have revealed that paracrine/autocrine signaling is involved in differentiation of a population of ASCs into adipocytes, and thereby that microfluidic perfusion cell culture systems are a powerful and a useful tool to investigate paracrine/autocrine signaling within a cell population in general. In addition, the results indicated that optimal concentrations of exogenously stimuli in the cell culture medium in perfusion cultures may be different to what is optimal in static cell cultures.

To enable more controlled experimental conditions and for further studies into adipocyte differentiation, it is necessary to be able to control the cell seeding density by an improved cell loading
method. Furthermore, a standardized method for collection of CM should be employed, as well as the CM must be freshly collected at use.

To further investigate the effect of CM on the adipocyte differentiation process, expression of the early markers of differentiation, such as the transcription factors C/EBPβ, C/EBPδ, PPARγ2 and C/EBPα, can be examined by RT-PCR in a time course study of the adipocyte differentiation. To obtain enough cells for RT-PCR, cells may be collected from several chambers.

Analysis of conditioned medium from differentiating ASCs vs. medium from non-differentiating ASCs by methods such as for example microarray technologies or mass spectrometry may be used to identify possible paracrine/autocrine signaling candidates. Our microfluidic cell culture system would then be a useful tool to screen possible candidates for influence on the adipocyte differentiation process.
Chapter 7

Overall Conclusion and Outlook

7.1 Conclusion

The main objective of this project was to evaluate the usability and applicability of microfluidic perfusion cell culture systems as a tool in biological research. Based on obtained working experiences through establishment of cell culture procedures and application of our developed microfluidic cell culture systems within stem cell research, usability and applicability have been investigated and evaluated.

Regarding system design, the evaluation of the tested microfluidic cell culture systems, developed in the ProCell project and the FAST group, has shown that the requirement to develop a system with integrated fluidic actuation, liquid reservoirs, liquid interconnections and cell culture chambers, which at the same time displays good properties regarding optics, compatibility with standard laboratory equipment, portability and usability, has been met. However, improvements regarding robustness and thereby reliability has been shown to be needed. As a biologist it is very important that the methods and tools are reliable and that the functional cell assays can be reproduced with a satisfactory result. The demonstrated issues regarding robustness and reliability are probably one of the reasons for the lack of implementation of microfluidic cell culture systems into biological/biomedical research laboratories.

Procedures to performed successful long-term microfluidic perfusion cell culture experiments have been established. Microfluidic perfusion cell culture of HeLa cells and ASCs showed satisfac-
tory cell attachment, morphology and proliferation and has been performed without contaminations by bacteria or fungi. However, further optimizations have to be done to improve the control of the cell seeding, which can be important in many functional cell studies. In addition to demonstrate satisfactory normal cell culture, we have applied our developed microfluidic perfusion cell culture system to demonstrate the potential of the system to regulate gene expression of a gene of interest by external actuation and with real-time microscopy detection.

Further successful application of the microfluidic perfusion cell culture system has been shown by investigation of ASC differentiation into adipocytes, where we have revealed that paracrine/autocrine signaling is involved in differentiation of a population of ASCs into adipocytes. In this study, we demonstrated that microfluidic perfusion cell culture systems are a more powerful and a useful tool to investigate paracrine/autocrine signaling within a cell population compared to static cell culture. Thus, we have been able to perform novel biological research by the use of our developed microfluidic perfusion cell culture system.

### 7.2 Outlook

In this project we have demonstrated that simple microfluidic perfusion cell culture chips are a powerful tool to investigate paracrine/autocrine signaling within a cell population compared to conventional cell culture methods. Investigations of paracrine signaling could be further expanded by co-culture experiments of two or more cell types either patterned in the same cell culture chamber or in different cell culture chambers coupled in series in different combinations. With respect to differentiation of ASCs into adipocytes, this approach could also be applied to further investigate the effect of high density cell cultures on the differentiation efficiency of low density cell cultures.

However, for the use of simple microfluidic cell culture chips with cells in a monolayer for study of the cellular response to different external stimuli to be advantageous compared to conventional cell culture in a well plate, the system has to provide possibilities for automated spatial and temporal control of cell culture conditions of many samples in the same experiment.

Another great potential of microfluidic cell culture systems lies in the possibility to create tissue-like environments. By micro- and nanotechnologies and biochemical engineering, matrices and gel structures can be created to provide structural support and ECM-like properties for the cells in a 3D environment, which is supplied with nutrients and other possible external stimuli through perfusion with cell culture medium. This could be useful for, for example, to create stem cell niche-like environments for co-culture of MCSs, endothelial cells and other stem cell niche cells. If such
**Overall Conclusion and Outlook**

*Vivo*-like cellular environments can be realized and later upscaled in a chip with many “tissue” samples, microfluidic cell culture would really distinguish itself from what it possible today with conventional cell culture methods.

However, as pointed out in this project, it is very important in the developmental process also to keep focus on usability and reliability. Furthermore, if more projects include application of the developed device by a biologist to investigate a biological issue, the results may be presented not only in the microfluidic research community, but also in the biological research community and thereby possible attract more interest for applying microfluidics for research into cell functions.
Appendices

Appendix 1

Effect of conditioned medium on adipocyte differentiation

a) Flow rate: 33 mL/min

b) Flow rate: 125 mL/min

Area of lipid-filled droplets normalized to total cell area vs. Days after onset of differentiation for different conditions and CM batches.
Fig. 45. Effect of conditioned medium on adipocyte differentiation. The ASCs were induced to differentiate at different flow rates, a) 33 nL/min, b) 125 nL/min and c) 500 nL/min in either AM or 1.5x AM+CM (a 1:1 mixture of 1.5 times the concentration of AM and CM collected from differentiating cells in static culture). Differentiation is measured by the total area of lipid-filled droplets using ImageJ and normalized to the total cell area at the start of differentiation. Differentiation is shown over time from onset of differentiation. For each pair of cultures differentiated in either AM or 1.5x AM+CM is indicated the batch of CM and whether it was freshly collected. The shown diagrams are from 9 independent experiments.
Appendices

Appendix 2

Effect of concentrations of adipogenic stimuli on adipocyte differentiation

**Fig. 46. Effect of concentrations of adipogenic stimuli on adipocyte differentiation.** ASCs were loaded with different cell suspension densities, $0.5 \times 10^5$, $2 \times 10^5$, $4 \times 10^5$ cells/mL, and induced to differentiate at a flow rate of 500 nL/min in AM, 1.5x AM+CM 1:1, AM diluted 4x or 1.5x AM diluted 4x+CM 1:1. All conditions were tested in one experiment by the use of a 16 chamber cell culture chip. Differentiation is measured by the total area of lipid-filled droplets using ImageJ and normalized to the total cell area at the start of differentiation. The results shown are from one experiment out two independent experiments. Results from the other experiment are shown in **Fig. 40.**

a) The differentiation efficiency shown at day 18 after onset of differentiation.

b), c) and d) The differentiation shown over time for the cell loading suspension densities $0.5 \times 10^5$, $2 \times 10^5$, $4 \times 10^5$ cells/mL, respectively.
Appendices

Appendix 3

Paper 1

Peder Skafe-Pedersen, Mette Hemmingsen, David Sabourin, Felician Stefan Blaga, Henrik Bruus, and Martin Dufva: A self-contained, programmable microfluidic cell culture system with real time microscopy access. Published online in Biomedical Microdevices, 13 December 2011.
A self-contained, programmable microfluidic cell culture system with real-time microscopy access

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Abstract Utilizing microfluidics is a promising way for increasing the throughput and automation of cell biology research. We present a complete self-contained system for automated cell culture and experiments with real-time optical read-out. The system offers a high degree of user-friendliness, stability due to simple construction principles and compactness for integration with standard instruments. Furthermore, the self-contained system is highly portable enabling transfer between work stations such as laminar flow benches, incubators and microscopes. Accommodation of 24 individual inlet channels enables the system to perform parallel, programmable and multiconditional assays on a single chip. A modular approach provides system versatility and allows many different chips to be used dependent upon application. We validate the system’s performance by demonstrating on-chip passive switching and mixing by peristaltically driven flows. Applicability for biological assays is demonstrated by on-chip cell culture including on-chip transfection and temporally programmable gene expression.

Keywords Programmable · Cell culture · Microfluidic · Portable · User-friendly · Modular

1 Introduction

Systems based on microfluidic technology have the potential to be an important future research tool within the cell biology field (Veléz-Casquillas et al. 2010; Wu et al. 2010; Yeon and Park 2007; Young and Simmons 2009). Among the advantages of microfluidics compared to conventional technologies are reduced sample consumption, portability, parallelization and increased spatiotemporal control of the sample environment (Liu et al. 2010; Park et al. 2010; Taylor et al. 2009; Veléz-Casquillas et al. 2010) leading to possibilities for assays not possible with today’s technology. Despite these advantages and the existence of highly advanced systems (Gomez-Sjöberg et al. 2007; Taylor et al. 2009) the microfluidic technology still has not made its entry as a common everyday tool into the biological research laboratories (Whitesides 2011; Young and Beebe 2010; Young and Simmons 2009). In order to fully get the microfluidic technology adopted by the biological society it is time to consider issues such as usability, availability and conformity to existing standards and protocols as pointed out elsewhere (Andersson and van den Berg 2006; Whitesides 2011).

As microfluidic cell culture is still a relatively young field of research, as a natural consequence the development of microfluidic systems has to a high degree been technology driven and focused on implementation and integration of functionality rather than usability and availability.

Often, microfluidic cell culture systems focus only on a subset of components or a single functionality without addressing issues of portability and connectivity to reagents.
and actuation hardware. Typical prototyping configurations include a microfluidic chip containing the actual cell culturing and manipulation functions combined with an external perfusion system based on bulk syringe (Hung et al. 2005; Kim et al. 2006; King et al. 2006; Stangegaard et al. 2006) or peristaltic (Wang et al. 2007; Zhang et al. 2009) pumps attached through glued or press fitted needles and extensive tubing. This configuration has advantages in terms of rapid prototyping and device testing without the need for development of pumping solutions. However, bulk pumps heavily restrict system portability and conflict with established working routines, increased required sample volume and limit the number of possible individual inlets to the microfluidic chips. Furthermore, the use of distantly placed pumps connected by extensive amounts of tubing can be prone to formation and trapping of bubbles. Approaches to avoid external pumps include integration of peristaltic pumps on chip using either pneumatic (Hsieh et al. 2009; Kim et al. 2008; Melin and Quake 2007; Yu et al. 2009) or Braille display (Gu et al. 2004; Tung et al. 2007) actuation. This has the advantage of allowing for a large number of pump lines and integration of reservoirs. However, the integrated approach can set restrictions on the possible assays due to a fixed design. Furthermore, actuation of pumps and valves is often still accomplished by large amounts of tubing to external solenoid actuators. Systems allowing for a higher degree of portability have been explored and demonstrated. Examples include a fully portable culturing solution (Futai et al. 2006) and a parallel culturing system with a simple actuation interface by a single pressure source (Sugiura et al. 2008).

Examples of approaches interfacing established standards include a passive culture array by Lindström et al. (2009) containing 672 micro chambers on a chip with dimension of a standard microscope slide. The chip can be seeded by FACS instruments and allows for high-throughput single cell analysis by conventional imaging equipment, but is not constructed for automated perfusion. Systems using the well plate standards include a microbiology fermentation system with easy interconnection and handling by Buchenauer et al. (2009). Lee et al. (2007) presented a 96 well plate based perfusion system optimized for existing equipment and protocols with simple but limited fluid control by gravitationally driven perfusion. Systems for high throughputs cell culture have been presented by Meyvantsson et al. (2008) and Puccinelli et al. (2010) combining passive well plate format microfluidics with external pipetting. This allows for a high degree of automation by fluid handling robots.

In this paper we present a stable, self-contained system, which is fully portable and compatible with common working routines of cell biology labs. Based on previously presented interconnection standards (Sabourin et al. 2010a) and improved and further miniaturized near-chip peristaltic micropumps (Skafte-Pedersen et al. 2009) the construction of the system facilitates handling without specialized skills in microengineering as exemplified by the rapid formation of 32 system-to-chip interconnections formed with only tightening four screws. Yet, it provides up to 24 inlets to one single passive chip and in this and other aspects outperforms other more advanced systems regarding the number of individual fluidic inlet lines (Gomez-Sjöberg et al. 2007; Taylor et al. 2009). The 24 inlets are pumped with three peristaltic micropumps each driving 8 liquid streams. The pumps are programmable in direction and speed over time using custom designed software to provide automated fluid handling. We validate the system by determining switch rate between one reagent and another and mixing in the passive chip. Finally we control and monitor gene expression in living cells.

2 Materials and methods

2.1 System base

The entire system is based on modular components previously described which negate the use for adhesive based permanent assemblies (Sabourin et al. 2010b). With few exceptions all components are custom made in-house by direct mechanical micromilling (Mini-Mill/3, Minitech Machinery Corporation, GA, USA). An overview image of the system with mounted culture medium vials is shown in Fig. 1 and a conceptual system component sketch including peripherals is given in the electronic supplementary material (ESI), (ESI Fig. 1). System components are assembled on a pair of 5 mm thick polycarbonate (PC) base plates. The outer base plate (OBP) rests on the microscope stage and supports the reservoir holders, electrical connector, and inner base plate (IBP). Inlet reservoir holders accommodate up to twenty-four 4 mL glass vials (13090222, La-Pha-Pack GmbH, Germany) with silicone/poly(tetrafluoroethylene) (PTFE) sealed screw caps (13150815, La-Pha-Pack GmbH, Germany) and outlet reservoir holders contain up to six 10 mL glass vials (18091306, La-Pha-Pack GmbH, Germany) with butyl/PTFE sealed screw caps (18031416, La-Pha-Pack GmbH, Germany). The IBP supports three peristaltic micropumps with motors, three inlet adaptors for connecting pumps and reservoirs through tubes, a combined outlet and cell loading block (OCLB) and a support frame for the microfluidic chip (Fig. 2). The IBP is mounted below the OBP in order to fit directly into the central recess of the scanning stage (Scanning stage 130×85 mot; CAN, Carl Zeiss, Germany) for secure mounting by a spring loaded click-on approach. The pumps have integrated, reversibly sealing ball joint interconnections with a self-aligning feature (Sabourin et al. 2010a). The same type of interconnections is integrated in the bottom of the OCLB. These
integrated ball joint interconnections, which are firmly secured by the IBP are used to interface the microfluidic chips directly to the OCLB and outlet side of the pumps, respectively. The 32 system-to-chip interconnections are established by snapping chips in place and tightening four screws, one in each corner of the chip support frame (Fig. 2). The self-aligning ball joint interconnections and corresponding inlet holes on the chip create interconnections with high sealing strength (Sabourin et al. 2010a). The inlet sides of the pumps have mounted inlet adaptors containing cylindrical cavities with a volume of ~30 μL. These can be used either directly as pipetting reservoirs for short assays or a connection base for connecting the pumps to the glass vials via PTFE tubing. In the latter case a piece of silicone tubing (1×3 mm, 228–0701, VWR, Belgium) is mounted in the hole to serve as a gasket for PTFE tubing (BOLA 1810–01, Bohlender GmbH, Germany). The outlet block is a combined structure of PC and poly(dimethylsiloxane) (PDMS) containing eight integrated ball joint interconnections and eight ~15 μL chambers, which serve as both socket for PTFE outlet tubing and reservoirs for loading up to eight different cell suspensions.

The entire system is enclosed in an incubator (Incubator XL Dark S1, Carl Zeiss, Germany) when mounted in the microscope. To limit the influence of gas permeable materials on the liquid the entire system is equipped with a flexible atmosphere cover during long-term cell culture. The cover is reversibly attached by Velcro® tape and fed with a
2.2 Chip design and fabrication

The employed microfluidic chips are based on PMMA (Plexiglas XT 20070, Röhm GmbH, Germany and Solaris Clear S000, PSC A/S, Denmark) and fabricated by micromilling followed by a UV assisted local heat bonding (Truckenmüller et al. 2004). In short, the individual layers of the chip were cleaned with 70% EtOH and Milli-Q water before being exposed to UV (DYMAX, 5000 EC with bulb 36970, CT, USA) for 90 s. Following exposure the layers were sandwiched between two glass slides in an alignment setup and bonded for 20 min at an initial bonding pressure of 2.3 MPa in a laboratory press (PW 10 H, P/O/Weber, Germany) pre-heated to 85°C. All chips have a total thickness of 3.5 mm and are, depending on the specific design, composed of individual sheets of PMMA ranging from 0.5 mm to 2 mm in thickness. The bottom layer of all chips is 0.5 mm for reduced optical path length from the sample to the objective. A total of 32 fluidic inlets and outlets are implemented as 800 μm holes in the top of the chip. Inlets are spaced 2.25 mm apart to conform with 1,536 microtiter well plate standards and placed along each chip side in groups of 8 to interface pumps and OCLB. The chip for cell culture and programmable gene expression (Fig. 3(a)) contains eight parallel fluidic networks each having three inlets meeting in a common intersection upstream a mixer and culturing chamber. The chamber has a footprint of 1.5 mm width by 4 mm length capped by isosceles triangles. Chamber height is 500 μm and inlet and outlet channels are connected at the top surface of the chamber.

Characterization of switching and mixing of liquids is made with chips containing four T-junctions with inlets routed from either the same or two neighboring pumps in order to test different flow combinations (Fig. 3(b)). Each mixer has an outlet channel length of 88 mm and a cross sectional area of 400 μm width and 150 μm height. Since the diffusive leak is dependent on the fluid velocity and thus intersection geometry the influence of geometry is tested through two variants of the intersection, one with 400 μm by 150 μm intersection and another with a constricted 200 μm by 70 μm intersection. 3D images of these can be found in ESI Fig. 2.

2.3 Pump construction

The pump construction is an upgraded and further miniaturized version of a previously presented work (Skafte-Pedersen et al. 2009) based on the three central components highlighted in Fig. 4. A peristaltic multi roller (MR) consisting of a central brass drive shaft encircled by eight stainless steel pins secured in nylon holders and connected to ball bearing end pieces occludes a monolithic PDMS microfluidic ribbon (μFR) with eight integrated channels against a static, supporting rotor bed (RB). The μFR is bookended by handling pieces in PMMA and integrated ball-joint interconnections for direct chip attachment.

The footprint of the central pump elements, including two sections for fluidic interconnections of all eight channels is 30×40 mm² and the total height is 20 mm excluding bolt heads. By rotating the MR, fluid volumes occluded between two pins are transported through the integrated tubing in the direction determined by the rotation of the MR. Due to a symmetric construction of the pumps their action is bidirectional with the same flow characteristics in either direction. A detailed description of pump operating principles is provided in Skafte-Pedersen et al. (2009).

For perfusion flow rates on the order of 100 nL min⁻¹ per fluidic channel low rotational speeds on the order of 10⁻¹ min⁻¹ are required. To achieve a smooth motion at such low speeds combined with a minimum footprint and weight for microscopy compatibility a geared stepper motor is used. The chosen model, which has a cross sectional area smaller than the remaining pump parts, is a 24 step miniature stepper motor (PRECIstep AM 1524, Faulhaber, Germany) equipped with a planetary precision gearhead of reduction ratio 152:1 (15A, Faulhaber, Germany). Including motor and
gearhead the total length of a complete eight-channel pump is 100 mm and the mass limited to approximately 50 g.

2.4 Peripheral components

A custom built control box powered by an external power supply (KY-05036S-12, Leadman Electronic Company, Inc., Taiwan) contains three stepper motor circuits (Stepper Motor Driver TA8435HQ CNC, Markus Mechatronics, Germany) and a USB I/O card (LabJack UE9, LabJack Corporation, CO, USA) as the main components. The system is connected to the control box through 12 electrical phases in a single ribbon cable terminated by a standard multipin connector. For long term cell culture the inlet and outlet reservoir caps are coupled with PTFE tubing (BOLA 1810–10, Bohlender GmbH, Germany) and pressurized with an air and 5% CO2 mixture through a sterile filter with a single Luer-Lok fitting.

2.5 Software control

The fluidic actuation is controlled by custom-built software based on Visual Basic for Applications (VBA) for direct integration of the pump control in the AxioVision microscopy software (AxioVision 4.8.2, Carl Zeiss, Germany). For advanced long term assays such as temporal cell programming requiring changes in perfusion settings over time, a module integrating each pump setting with a corresponding acquisition and automated image analysis sequence has been developed. The module is fed with an external MS Excel file containing the predefined perfusion, acquisition and convective-diffusive flux at the intersection and a down-stream pure convective flux at the outlet is employed to develop a steady state model. Combining this with the characteristic diffusion length \( l = \sqrt{2Dt} \) results in the following time-dependent estimate for the leak concentration expressed in terms of the characteristic leak ratio \( \chi \)

\[
\chi \equiv \frac{c_B}{c_1} = \frac{D}{D + v_1 \sqrt{2Dt}}. \tag{1}
\]

2.7 Analytical diffusive leak model

Due to the valveless construction of the chips there will be an inherent diffusive leak from the intersection when one or more inlets are stopped. An analytical 1D model based on the steady-state convection-diffusion equation combined with a characteristic diffusion time and length relation has been developed for design guidelines. If we assume a stagnant inlet channel to hold a solute with diffusion coefficient \( D \) and concentration \( c_1 \) connected to an infinite upstream reservoir of concentration \( c_1 \) we can find the diffusive leakage concentration \( c_B \) in the outlet channel subject to an average fluid velocity \( v_1 \). A constant concentration BC at the inlet combined with continuity in concentration and convective-diffusive flux at the intersection and a downstream pure convective flux at the outlet is employed to develop a steady state model. Combining this with the characteristic diffusion length \( l = \sqrt{2Dt} \) results in the following time-dependent estimate for the leak concentration expressed in terms of the characteristic leak ratio \( \chi \)

\[
\chi \equiv \frac{c_B}{c_1} = \frac{D}{D + v_1 \sqrt{2Dt}}. \tag{1}
\]
Using this as a design parameter it is clear that the diffusive leakage can be limited by increased velocity at the intersection and increased time. However, it should be noticed that the estimate is only valid for $\sqrt{2Dt_1} \leq x_1$, where $x_1$ is the distance from the intersection to the infinite reservoir.

Due to diffusion between the intersection and stagnant channel there will be a time lag when switching pump settings due to required flush of the contaminated stagnant channel. Assuming the intersection to be a constant source this lag can be estimated by the volume $V_p$ contaminated during stagnant conditions over time $t_s$ from the characteristic diffusion length $l_p = \sqrt{2D t_s}$. Based on this, the lag time $t_l$ required to flush the contaminated volume $V_p$ can be estimated to be

$$t_l = whQ^{-1} \sqrt{2Dt_s}.$$  \hspace{2cm} (2)

More in-depth derivations can be found in the ESI.

2.8 Numerical time-dependent switching model

2D FEM models are employed to determine the time dependence of the switching in a T-junction including the initial convection flushing phase and subsequent convection-diffusion balance leading towards a steady state leak. The models are implemented by a coupled steady-state Navier-Stokes and time-dependent convection-diffusion application mode. The model includes a volume force to take viscous stress from the top and bottom of the channel into account as used in the mixing models as well. For the model shown in Fig. 5 we use the following boundary conditions for the convection-diffusion equation: Inlet A has a constant concentration of unity representing an infinite reservoir and inlet B concentration of zero as the concentration of the flushing liquid. At the outlet the flux is assumed purely convective. All other boundaries are symmetry conditions. For the Navier-Stokes model, inlet B has a parabolic inlet velocity dependent on the flow rate. The outlet is a pressure outlet condition with no shear stress. All other boundaries are no-slip conditions.

As initial condition the upper inlet channel and the outlet channel are maintained at concentration $c=1$, whereas the lower inlet channel is at $c=0$ as illustrated in Fig. 5. The model containing a constricted junction assumes a constant channel height of 70 μm in the entire domain, since the switching dynamics are dominated by the convection-diffusion balance in the intersection. The convection-diffusion model is solved with a time dependent solver at time steps of 1 or 4 s for flow rates of 1,000 nL min$^{-1}$ or 250 nL min$^{-1}$, respectively.

2.9 Flow characterization

Flow patterns and characteristics of a separate pump as well as on-chip convection-diffusion dynamics were determined. For time-dependent flow pattern determination the outlet from individual channels of a peristaltic micropump were connected to a thermal anemometry volume flow sensor (Flowell, Flugent, France) by PTFE and polyetheretherketone (PEEK™) tubing (BOLA 1810–10, Bohlender, GmbH, Germany and Upchurch Scientific 1581, IDEX Health and Science, WA, USA) via an intermediate PMMA tubing connector chip. The inlet side of the pump was connected by PTFE to an open reservoir containing Milli-Q water by PTFE tubing and connector chip. Custom made software in Matlab R2010a (The MathWorks, MA, USA) was used to plot the pattern.

Determination of channel-to-channel and pump-to-pump variability were made in a separate test setup containing a chip with sixteen $1 \times 1 \text{ mm}^2$ channels addressed by two individual micropumps, where the advancing fronts of a water-based dye solution were used to determine pumped volume per full pump rotation. For this purpose and for durability tests another motor type (LEGO® Mindstorms®, Denmark) with higher rotational speed capabilities was used.

For on-chip characterization of switching rate and mixing capabilities using the chip shown in Fig. 3(b) the inlets in each T-junction were discriminated by feeding one inlet with a combined fluorescent and absorbing solution and the other inlet with an absorbing solution. For matched absorbance and excitation wavelengths this technique eliminates signal from the upper part of the channel by Lambert-Beer absorption as described by Bancaud et al. (2005). Fluorescein sodium salt (FS) (Sigma 46960-25 G-F) was used as fluorescent dye and Orange G (OG) (Sigma O3756-25 G) was employed as a matching absorbing dye. Concentrations of $c_{FS}=1 \text{ mM}$ and $c_{OG}=30 \text{ mM}$ were used to obtain the best compromise between fluorescent signal and decay length based on a parametric scan of concentration...
combinations (unpublished results). The two dyes were dissolved in a 50 mM carbonate-bicarbonate buffer solution (CBB) (Sigma C-3041) with a nominal pH of 9.6. As the nonfluorescent absorbing solution a 30 mM OG solution in 50 mM CBB was used to ensure identical absorption characteristics over the entire channel width. Based on the theory presented by Bancaud et al. (2005) this gives a 95% intensity reduction over 24 μm with the employed Zeiss EC Plan-Neofluar 10×/0.3 Ph1 objective.

The fluids for peristaltic pumping were contained in 4 mL glass vials connected by PTFE tubing (BOLA 1810–01, Bohlender GmbH, Germany) and the chip outlets were connected to 5 mL glass vials through PTFE tubing. Image acquisition software was AxioVision and automated analysis was performed by custom made software in Matlab with the aid of Bio-Formats (LOCI, UW-Madison, WI, USA) for reading the Zeiss ZVI file format.

2.10 Switching

For switching characterization a single T-junction was attached to two independent peristaltic pumps. The switching efficiency was evaluated by switching off the fluorescent inlet and turning on the nonfluorescent. Two different flow rates of 250 nL min⁻¹ and 1,000 nL min⁻¹ were used. Prior to switching, the system had been running at the initial condition for a minimum of 2 or 8 min at 1,000 nL min⁻¹ or 250 nL min⁻¹, respectively. The flow rates denote average flow rates.

Time-lapse series with a temporal distance of 2 s or 8 s at 1,000 nL min⁻¹ or 250 nL min⁻¹, respectively, were recorded using a 10×/0.3 Plan-Neofluor objective, 62HE filter, 470 nm Colibri LED light source and a Zeiss AxioCam MRm B/W camera. First image was acquired approximately 1 s before change of pump settings to ensure recording of initial condition. For each geometry and flow rate combination a total of three repetitions were made. Junction A was fed by a single peristaltic pump in phase, junction B was connected to two peristaltic pumps with arbitrary phase shift, junction C was connected to a peristaltic pump and a syringe pump (CMA 400, CMA Microdialysis, Sweden) equipped with 1 mL syringes (BD Luer-Lok Tip 1 mL, ref 309628, Becton, Dickinson and Company, NJ, USA) and junction D was operated by the same syringe pump. The nominal, average flow rate ratios of the inlets were 1:1 in all four junctions and the total flow rate was 250 nL min⁻¹ or 1,000 nL min⁻¹ per junction, respectively.

Image acquisition was performed using the same equipment as for switching experiments but measured at positions 0, 2, 4, 8, 16, 32 and 48 mm downstream the front edge of the intersection in order to determine the diffusive mixing as function of convection distance. At each position, a 20 s time-lapse series with 2 s intervals was acquired and repeated 2×3 times per setting for a total of 66 images in both bright-field and fluorescence per position per flow rate setting.

Mixing efficiency was quantified by the mixing index M as given by Lee et al. (2000). If we introduce the average pixel intensity I and the standard deviation of the sample σₓ on the pixel intensity the mixing index can be expressed as

$$M = 1 - \frac{\sigma_x}{I}. \quad (3)$$

The fluorescence images were used for statistical evaluation of the mixing index along a line across the channel. Optical edge effects were eliminated by centering and limiting the analysis segment to 360 μm and a manually determined background pixel value based on analysis of an OG filled channel was subtracted and the line segment smoothed before calculation of the mixing index for each individual image. Subsequently, the mean and standard deviation of the mixing index at each position and condition were calculated and plotted against the distance from the junction.

2.12 Cells and culture medium

HeLa Tet-On® Advanced cells (631155, Clontech) were cultured in DMEM/F-12 + GlutaMax™ (31331, Gibco) supplemented with 10% Tet System Approved Fetal Bovine Serum (FBS) (631106, Clontech), penicillin 100 U mL⁻¹, streptomycin 100 μg mL⁻¹ (P4333, Sigma), and geneticin...
with air supplemented with 5% CO₂ through a sterile reservoirs were coupled with PTFE tubing (BOLA on the cell culture chip to the IBP. Inlet and outlet with Milli-Q water to remove bubbles, before clicking the liquid reservoirs to the pumps were filled separately base plate. The cell culture chip and tubes connecting connections to pumps were assembled onto the system ized by autoclaving before use. Glass vials, tubing and Liquid glass vials, caps, and PTFE tubing were steril-

2.13 Microfluidic cell culture

Liquid glass vials, caps, and PTFE tubing were steril-ized by autoclaving before use. Glass vials, tubing and connections to pumps were assembled onto the system base plate. The cell culture chip and tubes connecting the liquid reservoirs to the pumps were filled separately with Milli-Q water to remove bubbles, before clicking on the cell culture chip to the IBP. Inlet and outlet reservoirs were coupled with PTFE tubing (BOLA 1810–10, Bohlender GmbH, Germany) and supplied with air supplemented with 5% CO₂ through a sterile filter. To avoid formation of gas bubbles a pressure of 0.3 bar was put on the flow system during the whole system preparation and cell culture period, only interrupted when for instance changing liquid reservoirs.

The connected flow system was sterilized by flushing with 0.5 M NaOH for 20 min at a flow rate of 4.5 μL min⁻¹, followed by washing with sterile water for 30 min at a flow rate of 4.5 μL min⁻¹ to remove all NaOH. The surface of the cell culture chambers was coated by passing a 50 μg mL⁻¹ polyethyleneimine (PEI) (408727, Sigma) solution in phosphate buffered saline (PBS) through the chip at a flow rate of 4.5 μL min⁻¹ for 15 min followed by 195 nL min⁻¹ for 1.5 h at room temperature. After coating, the flow system was flushed with cell culture medium for 20 min at a flow rate of 4.5 μL min⁻¹ before cell loading. Prior to cell loading the outlet tubes were removed from the OCLB.

HeLa Tet-On® Advanced cells were resuspended in cell culture medium added 0.025% w/v collagen (Sigma C3867) before about 10, Bohlender GmbH, Germany) and supplied of 37°C and 8% CO₂ to keep a pH of the cell culture supplies. The system was incubated in an atmosphere setting of 5×10⁵ cells mL⁻¹ of 5×10⁵ cells mL⁻¹ to remove all NaOH. The surface of the cell culture chambers was coated by passing a 50 μg mL⁻¹ polyethyleneimine (PEI) (408727, Sigma) solution in phosphate buffered saline (PBS) through the chip at a flow rate of 4.5 μL min⁻¹ for 15 min followed by 195 nL min⁻¹ for 1.5 h at room temperature. After coating, the flow system was flushed with cell culture medium for 20 min at a flow rate of 4.5 μL min⁻¹ before cell loading. Prior to cell loading the outlet tubes were removed from the OCLB.

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2.14 Regulated fluorescent reporter gene expression

DD-ZsGreen1-DR was cut out from pZsGreen1-DR (632428, Clontech) and cloned in-frame with the DD tag sequence in the multiple cloning site in pTRE-Cycle2 (631116, Clontech). Plasmid amplification was carried out in One Shot® TOP10 Chemically Competent E. coli (C4040, Invitrogen) cultured either in LB Broth (L7275, Sigma) or on LB agar (L7025, Sigma) added Kanamycin 50 μg mL⁻¹ (K1377, Sigma) or Ampicillin 100 μg mL⁻¹ (171254, Calbiochem). The plasmids were purified by using PureLink™ HiPure Plasmid DNA Purification Kit (K2100, Invitrogen) and diluted in TE-buffer to 100 ng mL⁻¹. HeLa Tet-On® Advanced cells passage 10 were loaded into the cell culture chambers on chip as described above. After 20 h of cell culturing and at a cell confluence of approximately 80%, the cells were transfected on chip. Briefly, 6.25 μg plasmid DNA (pTRE-Cycle2-ZsGreen1-DR) was diluted in 2.5 mL OptiMem medium (31985, GIBCO) and added 15.6 μL of Lipofectamine™ LTX (15338–100, Invitrogen) giving a proportion of DNA to Lipofectamine of 1:5. After gently mixing and incubation at room temperature for 25 min, the transfection complexes were diluted 1:3.2 in cell culture medium and then flushed into the cell culture chambers at a flow rate of 1.0 μL min⁻¹ for 20 min. The transfection process was performed without flow for 60 min, followed by a short flow period at a flow rate of 0.5 μL min⁻¹ for 10 min. These two steps were repeated for 6 h in total. Finally, the transfection complexes were removed by flushing with cell culture medium at a flow rate of 1.0 μL min⁻¹ for 20 min. Cell culture was continued at a flow rate of 0.5 μL min⁻¹ for another 18 h, before inducing mCherry and DD-ZsGreen1-DR gene expression by switch-ing to cell culture medium supplemented with doxycycline 0.5 μg mL⁻¹. DD-ZsGreen1-DR was stabilized by another switch to medium added doxycycline 0.5 μg mL⁻¹ and Shield1 0.5 μM and perfusion with doxycycline and Shield1 was continued for another 21 h. Lastly, a final switch was made to medium with doxycycline but without Shield1. Cell culture was continued for an additional 19 h before comple-tion of the experiment.

2.15 Imaging and image analysis

Time-lapse series of regulated fluorescent reporter gene expression were recorded every hour by a Zeiss Axio Ob-server.Z1 microscope equipped with a 10×/0.3 Plan-Neofluar objective, Colibri LED light source and a Zeiss Axiocam MRm B/W camera. A scan of 6×2 images, all acquired with a z-stack of seven slices (5 μm between each slice), were recorded for each chamber. The DD-ZsGreen1-DR signal was acquired at 470 nm excitation wave length through a 62HE filter, while mCherry was excited at 555 nm
and emission light acquired through a 43HE filter. DD-ZsGreen1-DR was exposed for 10 ms and mCherry for 500 ms. The images were processed by stitching the individual images together, converting the stitched images to one image and finally applying the AxioVision Extended Focus module on the z-stacks to obtain the best focused image. The fluorescent signal was quantified by calculating the densitometric sum (DS) based on the individual pixel values of the converted 16 bit image in the range between 1000 and 62955 for DD-ZsGreen1-DR and 1032 and emission light acquired through a 43HE filter. DD-ZsGreen1-DR was exposed for 10 ms and mCherry for 500 ms. The images were processed by stitching the individual images together, converting the stitched images to one image and finally applying the AxioVision Extended Focus module on the z-stacks to obtain the best focused image. The fluorescent signal was quantified by calculating the densitometric sum (DS) based on the individual pixel values of the converted 16 bit image in the range between 1000 and 62955 for DD-ZsGreen1-DR and 1032 and emission light acquired through a 43HE filter. DD-ZsGreen1-DR was exposed for 10 ms and mCherry for 500 ms. The images were processed by stitching the individual images together, converting the stitched images to one image and finally applying the AxioVision Extended Focus module on the z-stacks to obtain the best focused image. The fluorescent signal was quantified by calculating the densitometric sum (DS) based on the individual pixel values of the converted 16 bit image in the range between 1000 and 62955 for DD-ZsGreen1-DR and 1032–62439 for mCherry. Mean value of densitometric sum based on six individual chambers was calculated and normalized to the highest recorded value. The normalized averages and normalized standard error on the mean (SEM) for the regulated DD-ZsGreen-DR were plotted as function of time.

3 Results

3.1 System

The presented system is self-contained, modular and optimized for programmable long-term cell culture on microscope stages. The system is based on three peristaltic micropumps and a number of self-aligning interconnections (Sabourin et al. 2010a; Skafte-Pedersen et al. 2009). The central part of the system is an exchangeable cell culture chip equipped with 32 holes for fluidic input/output (I/O). Ball joint interconnections allowed the central microfluidic chip to be attached directly to 32 fluidic lines with a simple snap-on approach by securing four bolts between the chip support frame and the inner base plate. The system base plate is capable of holding up to 24 autoclaveable inlet reservoirs providing sufficient liquid for 5–10 days of cell culture and stimulation under typical conditions. With the exception of external pressure and power supply, which are attached via two standard connectors, all components and reagents are securely mounted and enclosed on a single, portable base, cf. Figure 1. Moving the system between workstation such as LAF benches, incubators and microscopes only required that the pressure source and the pump control cable were disconnected from the base plate. Disconnection and reconnection of the pressure source and control cable takes less than 1 min to perform. Because the fluidic connection from inlet vials to outlet vials via pumps and chip is not broken during disconnection of external control and pressure cables, this operation entails no risk of introducing bubbles during transport between work stations.

The base plate design with a lowered IBP ensured that the system was securely mounted in the microscope stage for long-term, multi position microscopy. The lowered IBP also allowed for use of objectives with low to medium working distance (WD). The compact size of the pumps combined with the direct chip attachment allowed the pumps and chip to fit between the condenser and objective of standard life science inverted microscopes. The current system is optimized for a motorized, inverted microscope (Zeiss Axio Observer.Z1, Carl Zeiss, Germany) with a motorized condenser with WD of 26 mm and objectives with a WD down to 2.5 mm.

3.2 Flow characteristics

The pumps are based on peristaltic occlusion of confined fluid volumes between the multi-roller pins and are therefore inherently pulsatile. Figure 6 shows an example of the flow pattern at an average flow rate of 0.36 μL min⁻¹. A distinct pulse pattern with typical pulse volumes of 0.08 μL is seen. Two pumps were tested to have average displaced volumes per full MR revolution ranging between 0.64 μL and 0.74 μL per revolution. The relative standard deviation in displaced volume between channels in individual pumps was tested to be between 2.8% and 8.7% similar to values for the previously reported pump (Skafte-Pedersen et al. 2009). Durability tests of the pumps were voluntarily stopped after 63,000 full revolutions. Using the minimum measured channel volume of 0.52 μL per revolution this corresponds to a pumped volume of approximately 33 mL per channel. For a typical culture experiment with average flow rates of 0.5 μL min⁻¹, the pumped volume corresponds to more than 45 days of continuous culture.

3.3 Switching

The system operates with passive chips, i.e. chips not containing any movable parts, and three pumps that each function as a closed valve when stopped. This means that liquid could be switched using T-junctions on the chip by stopping one pump and starting another. Since the chip did not contain a valve at the merging point of the liquid streams, it was necessary to determine the amount of diffusive leak from the stopped liquid line into the downstream line. Effects of switching were investigated for two flow rates

![Fig. 6 Excerpt of peristaltic pump pattern showing flow rate Q vs. time t at an average flow rate of 0.36 μL min⁻¹ measured with a thermal anemometry flow sensor. Typical pulse volumes are 0.08 μL. The dashed line indicates the average flow rate](image-url)
The experimental, numerical and analytical results of the passive on-chip switching are summarized in Figure 7. The diffusive leak ratio decreased more rapidly for the high flow rate than low flow rate for each cross section geometry and more rapidly for merging point with constricted cross section compared to large cross section. The finite bit depth of the employed camera (12 bit) and correction for a background, which changes slightly over the course of an experiment, put a limit on the possible resolution of the measured leak ratio. For this reason, only data points down to leak ratios of 1% have been included. 1% was arbitrarily decided to have no or little biological effect. At high flow rate and with the constricted cross section, the 1% leak was reached after about 15 s. For high flow rate and large volume cross section, 1% diffusive leak was reached after about 90 s. For lower flow rate the 1% diffusive leak ratios were observed after 0.7 and 7.2 min for constricted and large volume cross sections, respectively. Diffusive leak was determined by analytical and numerical models using a diffusion coefficient of \(D=6 \times 10^{-10} \text{ m}^2 \text{s}^{-1}\) and a stagnant channel length of \(5 \times 10^{-3} \text{ m}\). Diffusion coefficient value is based on data from Rani et al. (2005) scaled with temperature through the Stokes-Einstein relation. Table 1 summarizes the times for reaching 1% and 0.1% leak ratios based experiments and analytical and numerical models using the same modeling parameters as in Fig. 5. The analytical model uses the average fluid velocity based on flow rate and channel cross section. For all four combinations of geometry and flow rate a fair correlation between experiments and numerical and analytical models is observed.

Lag time due to upstream diffusive contamination after long-term stagnancy in the stopped inlet channel has also been estimated. The characteristic lag times \(t_l\) for flushing the inlet channel after long-term stagnancy was calculated from Eq. 2. With typical system values (\(D=6 \times 10^{-10} \text{ m}^2 \text{s}^{-1}\), \(w=400 \mu\text{m}, h=150 \mu\text{m}\)) the affected volume after 24 h is 0.6 \(\mu\text{L}\). Assuming a typical flow rate at switching of 0.25 \(\mu\text{L min}^{-1}\) (data not shown) and 1.0 \(\mu\text{L min}^{-1}\) (Fig. 8). These results indicate that the peristaltic micropumps essentially perform equal as pumps generating steady flow in terms of average mixing with the employed chip and conditions.

### Table 1: Time \(t\) to reach 1% and 0.1% diffusive leak ratio \(\chi\)

<table>
<thead>
<tr>
<th></th>
<th>Small intersection</th>
<th></th>
<th>Larger intersection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Q \text{ (nL min}^{-1})</td>
<td>(t_{1%} \text{ (s)})</td>
<td>(t_{0.1%} \text{ (s)})</td>
</tr>
<tr>
<td>Experimental</td>
<td>250</td>
<td>3.9( \times 10^1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Numerical</td>
<td>250</td>
<td>4.0( \times 10^1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Analytical</td>
<td>250</td>
<td>3.3( \times 10^1)</td>
<td>3.4( \times 10^3)</td>
</tr>
<tr>
<td>Experimental</td>
<td>1000</td>
<td>1.5( \times 10^1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Numerical</td>
<td>1000</td>
<td>0.8( \times 10^1)</td>
<td>2.3( \times 10^2)</td>
</tr>
<tr>
<td>Analytical</td>
<td>1000</td>
<td>0.2( \times 10^1)</td>
<td>2.1( \times 10^2)</td>
</tr>
</tbody>
</table>

Fig. 7 Experimental, numerical and analytical result for the leak ratio \(\chi\) vs. time \(t\), cf. Eq. (1), during passive on-chip switching in T-junctions with and without constriction. Experiments A correspond to the largest intersection and B to the smaller, constricted intersection, cf. ESI Fig. 2. Annotations 250 and 1,000 refer to the flow rate in nL min\(^{-1}\). Experimental data are averaged values ± standard deviation. Dashed lines represent numerical results and solid lines the corresponding analytical estimates. A diffusion coefficient of \(D=6 \times 10^{-10} \text{ m}^2 \text{s}^{-1}\) both analytical and numerical models and a stagnant channel length of \(5 \times 10^{-3} \text{ m}\) was used for numerical modeling.
3.5 Operability of the system

Assembly of the system takes place in a sterile condition of a LAF bench. The cells were introduced into the chips by loading a cell suspension into the embedded wells in the OCLB and sucking them in by reversing the pump direction. Due to the immediate vicinity to the culture chip combined with a vertical design, the cell loading wells eliminated the risk of cell loading failure due to unwanted upstream sedimentation before loading as can be experienced by for example loading from syringe pumps. Rapid input of cells in the system and increasing the viscosity of cell culture medium by the addition of collagen enhanced uniform plating densities along the chamber length.

Bubble formation and accumulation in the system was initially a serious problem for the low-permeable PMMA chips (unpublished results). This was solved by coupling the inlet and outlet reservoir caps with PTFE tubing and pressurizing the system with air supplemented with 5% CO₂ at 0.3 bar. After pressurizing the system, no air bubbles have been observed even for long perfusion times (3 weeks, unpublished results). In addition, the 5% CO₂ gas mixture improved maintenance of internal gas composition and thus pH of the medium. Since we observed no bubbles in pressurized systems, the need for on-chip bubble traps was eliminated, which eased initial chip filling, simplified chip design and manufacture and permits a greater number of other features, e.g. reaction chambers, to be incorporated within the area of the microfluidic chip.

3.6 Gene expression regulated by pump switching

The doubling time for the HeLa Tet-On® cells in the system was 32.5 h and in good correspondence with previously reported values for growth in conventional dish culture of unmodified HeLa cells and other microfluidic systemes (Petronis et al. 2006; Stangegaard et al. 2006). Figure 9 shows an example of the HeLa cells in one of the chambers 24 h after initial seeding.

We used the pTRE-Cycle2 tetracycline and Shield1 regulated gene expression system from Clontech to demonstrate the ability of the microfluidic system to temporally regulate gene expression by external actuation. HeLa Tet-On Advanced cells were cultured and transfected with pTRE-Cycle2-ZsGreen1-DR on chip. Gene expression of mCherry and DD-ZsGreen1-DR was induced by cultivating in medium supplemented with doxycycline 0.5 μg mL⁻¹. Regulated gene expression of DD-ZsGreen1-DR was demonstrated by switching to medium with or without 0.5 μM Shield1 in addition to doxycycline. As shown in Fig. 10, the fluorescent signal from DD-ZsGreen1-DR gene expression increased after switching to medium with Shield1 (turning on DD-ZsGreen1-DR gene expression) at approximately the same rate until it stabilized at about 19–21 h. After switching back to medium without Shield1 (turning OFF DD-ZsGreen1-DR gene expression), DD-ZsGreen1-DR was quickly degraded with a half-life of about 4.5 h. These kinetics were similar to DD-YFP expression in NIH3T3 cells (Banaszynski et al. 2006).

4 Discussion

4.1 System

The system was designed for usability and integration with microscopy. The basic design of the presented system comes from the library of components previously presented (Sabourin et al. 2010b). However, in addition to previously presented...
single functional components (Sabourin et al. 2010a; Skafte-Pedersen et al. 2009), this paper demonstrates how the interplay between improved versions of these components combined with application specific microfluidic chips leads to the creation of a robust system capable of performing biological assays with options of parallelization and automation. An example of such assays is the demonstrated real-time gene expression regulation controlled by the system.

Much of the usability of the system is due to the modularity of the individual components and how they are interfaced to the chip. A prime example is the formation of all the 32 interconnections between the systems and the chip by tightening four screws. A second advantage of the system is its portability, particularly between incubators, flow benches and microscope. This is enabled by incorporation of pumps, chips, and vials on common base plates. The only connections to the plate that is needed for operation is a single electrical cable equipped with a standard multi-pin connector and a Luer-Lok pressure connector. As the pumps function as valves when stopped the system can be decoupled from power and pressure and individual reservoirs changed in a LAF bench during long-term experiments, e.g. to limit degradation of fragile compounds with minimal interruption of constant perfusion assays. Due to the self-contained structure, risk of contamination during transport is furthermore minimized and the handling of the system is compatible with existing cell lab work routines and restrictions.

The modular approach for assembly of the system makes it possible to exchange a wide variety of the individual system components with the aid of a screw driver and without having to rebuild the entire system due to contamination, wear or failure of single components. This enhances yield and general usability of the system.

As the system is designed for automated long term cell culture with real-time microscopy it is important to ensure a stable environmental condition. The system itself only ensures control of the fluidic and thus biochemical near-cell conditions and therefore thermal and atmospheric control has to be established by other means. It has previously been demonstrated that on-chip thermal control can be utilized for stand-alone transmission microscopy of long term cell culture (Petronis et al. 2006; Stangegaard et al. 2006). Despite the advantages in terms of portability and demands on external equipment this feature, however, requires on-chip integration of the thermal components, which complicates fabrication, raises the costs of the culturing chips and can give unwanted optical problems such as autofluorescence or shadings. To minimize complexity and cost of the microfluidic system we employed a microscope mounted incubator (Incubator XL Dark S1, Carl Zeiss, Germany), which also ensures thermal stabilization of the microscope. This added feature is especially beneficial for limitation of focus drift over time.

The software is based on VBA and predefined settings for controlling pumps are made in Excel sheets. This

![Fig. 10](a) DD-ZsGreen1-DR gene expression was increased (turned ON) by switching to medium added Shield1 and decreased again (turned OFF) by switching to medium without Shield1. Doxycycline induces gene expression of both DD-ZsGreen1-DR and mCherry, but Shield1 regulates DD-ZsGreen1-DR at the protein level by protecting DD-ZsGreen1-DR from degradation. Annotations above micrographs indicate compound composition and annotation below indicate time points for switching to medium with Shield1 and later switching to medium without Shield1 are indicated with arrows. The fluorescent signal measurements are based on the densitometric sum ($DS$) of individual pixel values. Normalized by highest value ($DS_{\text{max}}$). Error bars indicate standard error of the mean based on six independent cell culture chambers in one experiment.

(b) Fluorescent signal of DD-ZsGreen1-DR gene expression plotted against time. Time points for switching to medium with Shield1 and later switching to medium without Shield1 are indicated with arrows.

This added feature is especially beneficial for limitation of focus drift over time.

The software is based on VBA and predefined settings for controlling pumps are made in Excel sheets. This
ensures an interface familiar to most users, reduces the requirements on programming skills and facilitates repetition of entire experiments simply by reloading the file. The simple software version decouples the fluidic actuation from the microscope and gives a more flexible, albeit manual, control of the system. Furthermore, it allows the system to be run from most Windows based computers making it suitable for portable applications and work in LAF benches.

4.2 Chips

The microfluidic chips contain only passive microfluidic networks and conform to the standard microscope slide format for easy integration with existing scanners, spotters and other slide handling equipment. Any fluidic control in terms of switching and pumping is handled by the three pumps which are immediately adjacent and in contact with the chip thus minimizing compliance problems. The volume displacement controlled pumping mechanism eliminates the requirements for balanced hydraulic resistance as is necessary for pressure controlled parallel flow systems. This eases the design of chips significantly and allows for rapid prototyping, e.g. with micromilling. The presented setup accommodates for a plethora of chip designs as long as the footprint and interconnections comply with the specified standard. Due to the non-specific mechanical attachment of PDMS interconnections the chip can be made of a variety of materials including, but not limited to, glass, silicon and thermoplastics. The micromilling based chip fabrication scheme used in this paper enables simple fabrication of multilayered, three-dimensional microfluidic networks allowing parallel, serial and combinatorial layouts. For chip fabrication on a larger scale, methods such as injection molding could be considered as an alternative method to produce the individual chip layers.

The chip employed for programmable gene expression distinguishes itself by both allowing for the demonstrated binary on/off situation from each of the inlets as well as a continuous spectrum of mixture ratios. This is possible because the peristaltic pumps both can act as valves and independently control flow rate. The speed of the respective pump is set in the Excel sheet and mixtures of two liquids can easily be established on chip. Depending on the application all three inlets can contain stimulants to be added in varied ratios. However, letting one of the inlets contain a buffer solute the system is readily converted into eight parallel temporal concentration gradient generators each with two stimulants and one diluter. By a proper control of the pumps the overall fluid dynamics near the cells can be maintained through a constant average flow rate while the concentration is varied through the flow rate ratio of the inlets.

4.3 Fluidic control for biological assays

Results on the externally actuated fluidic control showed that the time for switching from one liquid to another in the chip was between seconds to a few minutes (Table 1). The speed of exchanging a liquid from one to another is dependent on the flow velocity at the merging point and diffusion coefficient of the compounds. The flow velocity at the merging point can be increased by employing even smaller channels fabricated with other methods and set the pumps to run at maximum speed (volumetric flow rate 5 μL min⁻¹). It should be noticed that the leak ratio is measured in close proximity to the intersection and thus the downstream response, e.g. in a downstream cell culture chamber as in the design shown in chips such as those presented in Fig. 3(a), will have a natural delay due to channel residence time. The analytical estimate of lag in switch time after long-term stagnancy also shows time scales in the minute range, depending on channel geometry and flow rate. The switching performance compared to the presented results is thus not expected to be significantly reduced even after stagnancy periods on the order of days.

The pumps have a pulsatile flow pattern, which could be of concern for the on-chip fluid dynamics. However, for the employed chips and applications a comparison of models and experiments showed that for both switching and mixing, simple steady-state flow models can be used for design guidelines despite the actual peristaltic actuation. This suggests that the construction of similar systems can be based on corresponding steady-state modeling.

The time to switch one liquid for another with an acceptable contamination ratio is significantly shorter than the typical biological time scale of the presented assays. This has been demonstrated using on-chip transfected HeLa Tet-On cells where the microfluidic system is capable of controlling on-chip gene expression of mCherry and DD-ZsGreen-1-DR by externally mounted pumps. The good temporal correlation between change of pump settings and alteration of gene expression shows that the microfluidic system under the given culture conditions have a response that is sufficiently fast to resolve the time scale of the employed gene expression. The reversibility of the expression also demonstrates that the approach of passive microfluidic chips is a viable tool for this type of assays in that the diffusive leak as expected from the theoretical predictions is insignificant. This means that the use of integrated and more complex approaches is not necessary for these assays, and the experiments can be performed with high cost efficiency on passive, single-use chips.

Based on models and experiments we can thus conclude that passive on-chip networks for switching are performing satisfactory for such biological assays. The time scale of the gene expression in this experiment, which is also typical for
biological processes such as differentiation, cell division, metabolic response and migration, is longer than the fluidic switch time suggesting that the system can be used for a large variety of experiments.

Finally, the presented gene expression results are obtained by fully automated time lapse and the relatively small variation between the individual cultures demonstrate the power of multiple parallel microfluidic channels for gaining data for statistical analysis in a time and cost efficient manner.

4.4 Applications

The presented system is compatible with numerous biological applications. The particular application is tightly connected to the design of the chip attached to the system and the fact that the systems has three motors each controlling eight flow lines simultaneously. One configuration involves 24 chambers on the chip, where each chamber is controlled by one flow line. In such a configuration, it is possible to have well-controlled treatment of cells. Such configuration would for instance support research with the goal to understand signaling pathways involving a soluble compound released from cells or the impact of flow induced stress on the cell. These assays are now being performed in the system for investigation of stem cell differentiation (Hemmingsen et al. 2011), and examples of differentiating cells can be found in ESI Fig. 5 and ESI Fig. 6. In a 24 chamber configuration, it is not possible to obtain automated switching between factors, since the addition of factors to the cells is determined by what is added to the input vials. For temporal control, the system and chip must support feeding in factors to the growth medium over time. As demonstrated in Fig. 10 using the chip shown in Fig. 3(a), the system can support eight independent tests in parallel for temporal investigations. While the experiments shown are based on ZsGreen expression, it is possible to temporally control expression of any gene of interest cloned into a correct expression vector, in this case responding to the presence of doxycycline. Each test site (chamber) can be exposed to two different gradients simultaneously meaning that timely controlled expression of a gene (as driven by doxycycline concentration) can be combined with timely controlled presence of a soluble like cytokine. Due to the coupled actuation of channels by the three pumps, the number of different compounds that can automatically be added per chamber is in the present configuration limited to a maximum of three different compounds. For each chamber, however, these compounds can be unique and need not to be identical with compounds addressing neighboring chambers. Moreover, an expansion of the number of possible compounds can be implemented by adding compatible components capable of routing multiple inlets to the chambers (Conde et al. 2011). While the system is designed and validated for experiments involving higher eukaryotic cells, the system is not limited to such assays. For instance, we have validated that biochemical assays like DNA microarray analysis are supported by systems using the same components (data not shown). The presented system therefore would support research of for instance hybridization kinetics. Such analysis would increase the understanding of hybridization to and from immobilized probes.

5 Conclusions

We have devised and characterized a cell culture system based on peristaltic micropumps and passive polymeric microfluidic chips. The system allows for easy handling, loading, culturing and stimulation of cells with real-time optical detection. All handling can be performed without skills in micromechanics and components in contact with compounds and cells can be reused or discarded at a low cost through the use of passive microfluidic chips combined with off the shelf reservoirs on a modular system base. The portability and standardized construction of the system conforms well with established work flows and routines in standard cell culture labs. Biocompatibility and temporally controlled exposure has been demonstrated through programmable gene expression. By combining peristaltic pumping with a pressurized system we have eliminated the need for bubble traps in a low-permeable PMMA chip while retaining the advantages of a volume controlled pumping mechanism. The use of PMMA chips with a well defined geometry enables high-quality microscopy with various contrast and fluorescence techniques and ensures a low-permeable culturing surface amenable to coating.

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References

Appendices

Appendix 4

Paper 2

David Sabourin, Peder Skafe-Pedersen, Vasile Coman, Mette Hemmingsen, Jesper Petersen, Jenny Emneus, Jörg P. Kutter, Detlef Snakenborg and Martin Dufva: **MainSTREAM**: a modular and scalable microfluidic platform. In preparation.
MainSTREAM: a modular and scalable microfluidic platform

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Standardization and simplification of methods to construct microfluidic and use system would greatly benefit end users. In this report we explore a holistic approach to building microfluidics system. The approach is based on a microfluidic component library and design principles that simplify construction and use of microfluidic system. The library of components includes a miniaturized 8–channel peristaltic pumps, valves and sample-to-waste liquid management and provides means to connect these components with each other or to chips in a standardized fashion. Constructing various systems supporting perfusion cell culture and solid phase assay like DNA hybridizations and in situ hybridization tested the library of components. The results showed that 1) constructing diverse systems is aided by a standardized component approach, 2) establishing numerous interconnections to components and chip is aided by handling eight fluidic lines as aligned blocks instead of handling eight fluid lines individually, 3) highly usable systems required that all liquid connections are fixed in space on a frame to which a chip can be attached to, 4) highly usable systems required that all pumps, valves and liquid management is collected on a portable, 5) the library provides excellent biocompatibility in terms of cell culture and cell differentiation, 6) the library can automate multistep processes like in situ hybridization. In conclusion, the library of components described here and designated MainSTREAM, supports rapid construction of user-friendly microfluidics systems as well as biological experiments carried out in these systems.

Microfluidic, Platform, Modular, Pumping, Valving, Multiplexing
Introduction

Microfluidics offers many potential advantages over traditional techniques and can therefore enable new research and approaches in a variety of fields such as cell biology, structural biochemistry and analytical chemistry (Whitesides 2006). However, practical concerns including reliability, accessibility, affordability, portability, ease of use, assembly, integration and control of microfluidic devices hinder usability and, ultimately, adoption of microfluidic approaches (Fredrickson and Fan 2004; Andersson and van den Berg 2006; Whitesides 2006; Young and Beebe; Young and Simmons; Whitesides 2011). It is possible that the reason for the existence of these practical concerns is that there is little engineering research addressing microfluidics on the system level. There is in contrast abundant and excellent literature describing function of only one part of a system, i.e the chip. We hypothesize that the majority of the practical concerns raised above must be addressed on the system level rather than the single component level such as the chip. We define a system as every component to operate a chip as intended including pumps, valves, computer controllers, reagent vials, waste containers and means to connect these components together and together with a chip. In this paper we explore how components can be designed and be connected together into systems that addresses the majority of the practical concerns raised above.

Modular microfluidic systems of either academic or commercial origin (www.epigem.co.uk; www.labsmith.com; www.thinxxs.com; Grodzinski, Yang et al. 2003; Pepper, Palsandram et al. 2007; Rhee and Burns 2008; Sun, Wang et al. 2008; Yuen 2008; Yuen, Bliss et al. 2009) are influenced by both LEGO® and electronic breadboards aim to provide methods to rapidly assemble sealed microfluidic networks by simplifying interconnection formation between microfluidic chips. Additionally, modular systems present easily reconfigurable elements that can be assembled quickly and allow flexibility in design and construction of fluidic networks (Rhee and Burns 2008; Yuen, Bliss et al. 2009; Mark, Haeberle et al. 2010). Though modular approaches simplify interconnection formation, they fail to address and resolve fluidic actuation, routing, control and sample to waste liquid management (www.epigem.co.uk; www.labsmith.com; www.thinxxs.com; Grodzinski, Yang et al. 2003; Pepper, Palsandram et al. 2007; Rhee and Burns 2008; Sun, Wang et al. 2008; Yuen 2008; Yuen, Bliss et al. 2009). The resulting systems consist of chips connected to, for example, a syringe or peristaltic pumps. Such systems are very hard to place near or within detection instruments and incubators. Extended tubing then required to interface to microfluidic chips result in large dead volumes, induce compliance, interfere with detection and limits the portability of the systems. This suggests the next step to increase system level performance and usability is to provide scalable means to integrate pumps, valves and sample-to-waste liquid management within a single movable microfluidic unit.

A potential and commonly implemented solution to the above is the integration of fluidic actuation and routing functions into the microfluidic device. Multi-layer elastomeric approaches including microfluidic Large Scale Integration (mLSI) (Unger, Chou et al. 2000; Gomez-Sjoberg, Leyrat et al. 2007; Taylor, Falconnet et al. 2009) and Braille display activated microfluidic chips (Gu, Zhu et al. 2004; Futai, Gu et al. 2006; Tung, Torisawa et al. 2007) are examples of this approach and offer large multiplexing capabilities. However, the actuators, external pressure sources, solenoids, computers, and reagent vials and waste vials required to drive mLSI systems are still connected to the chip via tubes and cables. Again this limits the portability of the approach (Mark, Haeberle et al. 2010). Braille display (Gu, Zhu et al. 2004; Futai, Gu et al. 2006; Tung, Torisawa et al. 2007) approaches may overcome portability issues but uptake of this approach has been slow and is attributed to the complexity of the equipment and the unconventional nature of the platform (Young and Beebe 2010). Sample-to-waste liquid management systems are not easily integrated using mLSI and Braille display based approaches. While sample-to-waste liquid management seems trivial, it requires an array of sample holders, from µL to mL in size, to be directly connected to the chip ideally without any tubings. Combining µm scale features of pumps and valves with the cm scale of sample holders on the same chip is not necessary a simple task.

Another solution to the raised issues of microfluidics is addressed in the point of care (POC) system (Mark, Haeberle et al. 2010). POC systems aim to be highly portable, user friendly and typically consist of a custom control box which runs cartridges. The cartridges usually contain the reagents and components required for detection and quantification of analytes as well as sample ports.
The control box contains the actuators required to move liquids within the cartridge as well as detection units and a user interface. POC systems are mainly designed for repetitive, standardized, high volume assays, characteristics which is what is required for microfluidics to move into mainstream use in biomedical laboratories. However, adaptability of such systems is poor when it comes to variety of chips that can be processed and interfacing with various detection equipments.

With the above in mind, we asked ourselves if it was possible to find a method and design components that allow engineers to construct complete microfluidic systems which are scalable, flexible, portable, reliable and easy to use and which also resolve the previously described practical issues. In particular, the methods and components should provide i) simple and reliable sample-to-waste interconnections, ii) numerous fluidic inputs and outputs to chips, iii) pumping and iv) routing/valving capacity for these inputs, and a high degree of v) portability, vi) reconfigurability/flexibility in system design, vii) compatibility with detection equipment, and viii) usability in terms of robustness, biocompatible and compatible with biological work procedures. The resultant microfluidic platform presented here is designated as MainSTREAM.

MainSTREAM evolved from two previously described achievements, independently addressing interconnection and pumping methods, into a component library which collapses microfluidic systems inclusive of chips, reagent and waste vials, pumps and valves, into compact and portable experimental units without sacrificing flexibility and scaling of microfluidic system construction. Through genotyping, cell culture and in situ hybridization (ISH) applications we demonstrate that the platform’s component library is a versatile tool set for building microfluidic systems with applicability to a broad range of microfluidic applications within the field of biomedical research. Finally, the reliability and usability of system build with MainSTREAM components was tested.

Materials and Methods

General fabrication

Unless otherwise noted, all MainSTREAM components, and elements thereof, were based on CNC micromilled components (Skafte-Pedersen, Sabourin et al. 2009; Sabourin, Snakenborg et al. 2010). Multi-layer PMMA chips were bonded via a UV-assisted bonding process (Truckenmuller, Henzi et al. 2004). Bonding faces of the PMMA chips were exposed to UV (Dymax EC 5000 with p/n 36970 bulb, Torrington, Connecticut) for 1 min. and were subsequently bonded between glass plates in a bonding press (P/O/Weber, Remshalden, Germany) at 85°C for 10 minutes at an initial applied pressure of approximately 8 kN for chips measuring 26 mm x 76 mm (l) and 13.50 kN for chips measuring 52 mm x 76 mm. PDMS (Sylgard 184, DowCorning) used for elastomeric components was mixed in a 10:1 mass ratio of elastomer to curing agent and placed under vacuum to remove air bubbles. PDMS components were cured overnight at 65°C.

Microfluidic ribbons

Type I µFRs (Figure 1a) are designed for use in the micropump (Figure 2) and camshaft valve (Figure 3) and connect microfluidic elements found on both sides of the micropump and valve. Type I µFRs are monolithically cast from PDMS by a method previously described (Skafte-Pedersen, Sabourin et al. 2009; Sabourin, Snakenborg et al. 2010) and contain eight integrated circular 240 µm diameter channels spaced 2.25 mm centre-to-centre (1536 well plate standard). The raised tubing-like features (Figure 1b and c) of the Type I µFRs are occluded during micropump and valve operations. Type II µFRs (Figure 1e) are shorter versions of the Type I µFR. All µFR channels end with ball joint features (Figure 1d), which provide self-aligning, minimal dead volume interconnections (Sabourin, Snakenborg et al. 2010). Polycarbonate (PC) brackets, either square or round in shape, (Figure 1a and 1e) are placed into the µFR moulds prior to PDMS casting and, during curing, are integrated into the µFR. Overall dimensions, inclusive of the PC brackets of the Type I and II µFR are 48 mm (l) x 30 mm (w) x 5 mm (h), and 20 mm (l) x 30 mm (w) x 5 mm (h) respectively.
**Micropump**

*MainSTREAM*’s micropump (Figure 2) is a further miniaturized and improved version of a multi-channel, mechanically actuated peristaltic pump previously described (Skafte-Pedersen, Sabourin et al. 2009). The three main parts of the micropump are a multi-roller (MR), a rotor bed (RB), and a Type I µFR (Figure 2a). The MR is made from aluminium blocks which house 4 mm inner diameter (ID) ball bearings (VXB Bearings, Anaheim, USA). Nylon discs mounted on a central brass shaft align eight free rolling 2 mm stainless steel pins. The RB is made from polycarbonate (PC). When attached to the micropump, the RB’s shape results in occlusion of the Type I µFR tubing features between the RB and the MR’s rolling pins. This enables peristaltic pumping. The footprint of an assembled micropump with Type I µFR attached to microfluidic chips excluding motors is 30 mm (w) \times 40 mm (l) \times 20 mm (h).
seen middle-left protruding from between the RB and MR. Integrated channels of the Type I µFR filled with dye to facilitate visualization

Valve component

MainSTREAM’s camshaft valve (Figure 3) also uses a Type I µFR (Figure 1a). The camshaft (Figure 3a) is fabricated from the same parts as the micropump’s MR except that the stainless steel pins are replaced with individual cams that are stacked and aligned along a central 4 mm brass shaft and 2 mm alignment pins running between the nylon discs. Cams (Figure 3b) are made from aluminium and have a radius of 4.66 mm, a flat cut face, a 4 mm diameter central hole and 2 mm through holes. The 4 mm diameter central hole allows the cams to be placed on the camshaft. The 2 mm through holes allow flat cut faces of cams to be aligned at defined angles and positions relative to each other.

Fig. 3 Camshaft valve (a) Assembled camshaft valve. Individual Type I µFR channels are alternately filled with blue dye, green dye, water and red dye to facilitate visualization. (b) Schematic representation of the 3 different cams used to build the camshaft used in this report. The central hole in each cam is used for mounting these on a central shaft. The two smaller holes in each cam permit alignment of the cams and their flat cut face features at set angles to each other. (c) Schematic representation of valve operating principle. When the flat cut face is held parallel to and above a µFR channel, the channel remains open. Rotation to any point where a circular portion of the cam is above the channel results in depression of the PDMS directly underneath the disc and closing of the channel

To operate as a valve component, the µFR is aligned under the camshaft on a base plate via its two PDMS blocks (Figure 1a). A camshaft is then attached to the base plate. The height of the camshaft is set so that when the flat cut face of a cam is parallel to a µFR channel, the channel remains open (Figure 3c). Rotation of the camshaft to a position which places any circular portion of the cam above a channel results in contact between the cam and the µFR channels. This depresses the tubing wall and closes the channel. The aluminium cams are machined to thicknesses so that each cam is centered directly above each of the µFR’s 8 channels (Figure 3a).

The camshaft used in this report contains eight cams (Figure 3a). The cams were arranged such that each 45° degree rotation of the camshaft only allows flow through only one of the µFR’s eight channels at a time. The 45° steps continue in the same direction sequentially along the camshaft. If the starting camshaft position is assigned as 0° and having channel 1 of the µFR open, sequential 45° camshaft rotation steps in the same direction as the 45° steps of the cams’ flat faces will permit flow through only channel 1, then only channel 2, then only channel 3, etc., up to channel 8. Prior to operation, the valve’s camshaft was manually aligned to a pre-determined position.
Microfluidic chips

Microfluidic chips (Figure 4a) must contain the 10 hole patterns matching that of the µFRs (Figure 1). The inner 8 of these 10 holes are inlet or outlet holes with similar diameter and spacing as the ball joints (Figure 1d), 0.8 and 2.25 mm respectively. The outer two holes are 2 mm in diameter and allow µFRs to be fastened to microfluidic chips via 2 mm screws. Connector chips (CCs) are used for linking µFRs together (Figure 4) and are made from 1.5 mm thick PMMA and measure 30 mm (l) by 5 mm (w). The previously described 10 hole pattern is centered within these dimensions.

Fig. 4 (a) Sample microfluidic chip used in MainSTREAM systems. The 10-hole interfacing pattern is shown in the dashed box. (b) Connector chip (CC) with 10-hole pattern used to join two µFRs together. (c) CC (arrow) is between and used to connect Type I µFR from micropump (left) to Type I µFR from camshaft valve (right)

Sample to waste liquid management

Well arrays (WAs) (Figure 5a and b) are used to store reagents, samples and waste material. The WA chips also contain the 10 hole pattern in order to interface to a seal against a µFR. For applications requiring small volumes multi-layer PMMA or PC-based WAs were used (Figure 5a). For applications requiring larger volumes WAs were made from layers of PMMA sealed by intermediary PDMS gaskets (Figure 5b). When users specify the use of sample vials rather than WAs, tubing connectors (TCs) (Figure 5c) are used to connect up to sample vials to microfluidic systems. TC construction details are provided in the supplementary information, but in short they are a modified version of o-ring based interconnection blocks (Sabourin, Snakenborg et al. 2009) which also incorporate press-fitting of PTFE tubing into their PDMS portion and a second polymeric component to which interfaces to the PDMS interconnection features of the µFRs.

Fig. 5 Liquid Management. (a) Well array (WA) chip for small sample volumes, approximately 15 µL is seen attached to one side of a micropump. (b) A larger WA chip capable of holding volumes upwards of 4 mL is seen attached to two micropumps. (c) Tubing connectors (TCs) (in dashed box) connect tubing from sample vials to Type I µFRs. (d) Cell loading chip (CLC). The monolithically cast PDMS insert (dashed box) contains integrated wells bookended by sealing features which allow direct attachment to microfluidic chips and facilitate loading of cells into microfluidic systems

For cell culture applications a cell loading chip (CLC) (Figure 5d) is used. The CLC contains a PDMS portion integrated within a PC frame. The PC frame contains inset nuts which facilitate attachment to other components. The PDMS portion is monolithically cast as previously described
In this case 1.5 mm diameter pins are used to create eight wells with volume of approx. 15 \( \mu L \) each. The wells are bookended with o-ring sealing features (Sabourin, Snakenborg et al. 2009) on the bottom and top side. A lidding piece of PC is fastened and sealed against the top of the CLC with screws. This lidding piece contains either a single or eight press-fit PTFE tubings to collect waste liquids. To introduce cells into a microfluidic system, the top piece is unfastened by removing screws. Cells can then be loaded into individual wells and the top piece reattached.

**System Assembly**

All MainSTREAM components interface with each other using 2 mm screws and nuts. Base plates (Figure 6), made from PC, contain arrays of 2 mm holes in which 2 mm screws are inserted to position micropumps and camshaft valves in place. Base plates also contain recesses matching microchip dimensions to guide placement of chips within systems. In some cases, 2 mm nuts were press fit into undersized recesses within the baseplate to facilitate attachment of MainSTREAM components to each other. Brackets complimentary to the shape of \( \mu FR \)’s end brackets are sometimes attached to the baseplate adjacent to micropumps or camshaft valves. These guide \( \mu FR \) end bracket placement prior to the attachment of the \( \mu FRs \) to other components.

**Fig. 6** Sample MainSTREAM microfluidic system assembly scheme. (a) MainSTREAM components required for system assembly. Soft elements including Type I \( \mu FRs \) used in the micropump and Type II \( \mu FRs \) are in left hand box. Type II \( \mu FRs \) are shown at bottom left. Hard elements including microfluidic chips and well arrays (WAs) are in the right hand box. (b) The micropumps’ MRs are attached to the base plate. (c) A WA is placed on the left side of the MRs and a test chip is placed to the right. (d) Type I \( \mu FRs \) are connected to the chips and bridge the MR. (e) The RBs are attached to the MRs. Micropump assembly is now complete. (f) Remaining test chip and WA(far right) are placed in system. (g) Type II I \( \mu FRs \) are used to connect the microfluidic chips to the right of the pumps together. (h) LEGO® motors are mounted to the shafts of the micropumps. (i) The controller unit is attached to the motors. Samples are loaded into the WAs found to the left of the micropumps.

**Actuation and control of micropump and camshaft valve**

For microfluidic networks requiring greater levels of automation or for assays of longer duration, the LEGO® Mindstorms® NXT 2.0 robotics kit(www.LEGO.com) was used (Figure 6, supplementary video 1 and 2). The LEGO® kit provides multiple servo motors with 1 ° angle control, a controller (NXT Intelligent Brick), software interface (powered by National Instruments LabView®) for programming of motors, and other required components such as connector cables and power source. The micropump’s and camshaft valve’s shafts were shaped to attach to the LEGO® Mindstorms® motors. For assays not requiring automation, a hand crank (Figure 7, supplemental...
At conventional cell culturing the cells were incubated at 37°C and 5% CO2. Human Umbilical Vein Endothelial Cells (HUVEC) (Lonza) were cultured in M-200 Medium (M200500, Invitrogen) supplemented with 10% v/v FBS (04-2007-1A, Biological Industries), and penicillin 100 U mL-1, and streptomycin 100 µg mL-1. Rat adrenal pheochromocytoma cells (PC12) (ACC 159, DSMZ GmbH) were cultured in DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 15% v/v Horse serum (FBS) (631106, Clontech), penicillin 100 U mL-1, and streptomycin 100 µg mL-1 (P4333, Sigma). Human umbilical vein endothelial cells (HUVEC) (Lonza) were cultured in M-200 Medium (M200500, Invitrogen) supplemented with 10% v/v FBS (04-007-1A, Biological Industries), and penicillin 100 U mL-1, and streptomycin 100 µg mL-1. At conventional cell culturing the cells were incubated at 37°C and 5% CO2.

Genotyping in MainSTREAM

Hand crank driven micropumps were used to genotype patient samples for mutations in the human β-globin gene [24] (HBB) via an allele-specific hybridization assay. Microfluidically-addressable microarrays were incorporated within PMMA chips using a UV-activated poly(T)-poly(C) probe tag as described in a previously [24]. A PMMA genotyping chip sized identically to a microscope slide and containing 16 microfluidic lanes (see Figure 7) was interfaced to two micropumps. Each lane of the chip measured 1.5 mm (w) x 0.25 mm (h) x 21 mm (l) and was bookended by 0.8 mm inlet and outlet holes. Each lane contained DNA arrays constructed from poly(T)-poly(C)-tagged probes. The DNA microarrays allowed allele-specific hybridization (ASH) genotyping of three mutations in the human β-globin (HBB) gene [24]. The probe pairs used for ASH genotyping of these three mutations are provided in the supplemental information. Target preparation from patient archive material and washing buffers for the genotyping experiments were prepared as previously described [24] and were transferred from a loading chip through the pump and into the PMMA genotyping chip by turning the hand crank (Figure 7). Solutions were sequentially introduced into the PMMA chip. Prior to the introduction of the next required solution, the solution within the chip’s lanes was pumped out and absorbed dry with a paper towel. Approximately 12 µL of each solution was used at each step. Channels were first filled with 5 µL of 0.1 × saline-sodium citrate (SSC) supplemented with 0.5% sodium dodecyl sulfate (SDS) and incubated for 10 minutes to solubilize probes not bound to the surface after the spotting process. The channels were washed with additional 5 µL wash of 0.1 × SSC supplemented with 0.5% SDS in order to remove unbound probes. Fluorescently Cy3-labelled patient materials were prepared by PCR amplification and subsequent T7 in vitro transcription. The resulting amplified RNA were diluted 1:1 in hybridization buffer (10 × SSC supplemented with 1% SDS) (Sabourin et al 2010) Hybridization was performed at 37°C in a dark humid chamber for 2 h. The hybridization solution was then removed to prepare for multi-salinity gradient washing. Multi-salinity gradient washing allows genotyping via parallel analysis of identical arrays treated with different stringency wash buffers (Petersen et al 2009). In this case, each of the four lanes used to genotype a single patient was filled with one of the following four washing buffers: 2.0 × SSC, 0.55 × SSC, 0.10 × SSC and 0.035 × SSC, all supplemented with 0.1% SDS. These buffers correspond to 331.0, 91.6, 17.3 and 6.6 mM Na+ respectively. Lanes were filled for 1 min at room temperature to remove excess and unbound target. Following this the lanes were filled with the wash buffers and left for 30 min at 41°C. Hybridization reactions were imaged using a Zeiss Axio Observer.Z1 microscope. Identical focal and camera setting were used for all images collected. All signals were analyzed with GenePix Pro 6.1 (Molecular Devices, Sunnyvale, CA, USA) and analyzed according to the multi-salinity gradient washing method, which allow for the use of probes with different washing buffer optima in the same microarray (Petersen et al 2009).

Cells and culture medium

HeLa Tet-On® Advanced cells (631155, Clontech) were cultured in DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 10% Tet System Approved Fetal Bovine Serum (FBS) (631106, Clontech), penicillin 100 U mL-1, streptomycin 100 µg mL-1 (P4333, Sigma), and genetin (G-418) 100 µg mL-1 (11811-023, GIBCO). Adipose stem cells (ASC) (donated by Philippe Collas, University of Oslo) were cultured in DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 10% v/v Foetal Bovine Serum (FBS) (04-007-1A, Biological Industries), penicillin 100 U mL-1, and streptomycin 100 µg mL-1 (P4333, Sigma). Rat adrenal pheochromocytoma cells (PC12) (ACC 159, DSMZ GmbH) were cultured in DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 15% v/v Horse serum (HS) (H1138, Sigma), 3% v/v Foetal Bovine Serum (FBS) (F9665, Sigma), Hepes 0.5% v/v (H0887, Sigma), penicillin 100 U mL-1, and streptomycin 100 µg mL-1 (P4333, Sigma).
Cell culture in MainSTREAM

A PMMA microfluidic chip containing sixteen separate microfluidic cell culture chambers was placed in a MainSTREAM two-pump configuration. PMMA was used due to its biocompatibility [26]. The microfluidic chip was made from micromilled PMMA and was bonded as described above. Each of the sixteen cell culture chambers measured 1.5 mm (w) x 4.5 mm (l) x 0.5 mm (h) and were individually addressed. The LEGO® Mindstorms® kit was used to control and drive the micropumps addressing this chip. All flow rates described below are average flow rates. Liquid glass vials, caps, and PTFE tubings (BOLA 1810-01, Bohlender GmbH, Germany) were sterilized by autoclaving before assembly of the system. Tubes connecting the liquid reservoirs to the pumps and the cell culture chip were filled separately with Milli-Q water to remove bubbles, before connecting the cell culture chip to the pumps. To avoid bubbles, a pressure of 0.3 bar was put on the flow system during the whole system preparation and cell culture period, only interrupted when changing liquid reservoirs. The pressure was applied to the system by coupling inlet and outlet reservoirs with PTFE tubing (BOLA 1810-10, Bohlender GmbH, Germany) supplied with air supplemented with 5% CO2 through a sterile filter. The assembled microfluidic system was first sterilized by flushing with 0.5 M NaOH for 20 minutes at a flow rate of 5.2 µL/min. The chip was then flushed with sterile water for 30 minutes at a flow rate of 5.2 µL/min to remove all NaOH. A 20 µg/mL Laminin (L2020, Sigma) in phosphate buffered saline (PBS) solution was then passed through the chip at a flow rate of 5.2 µL/min for 15 minutes, followed by 250 nL/min for 45 minutes in order to coat the surface of the cell culture chambers. During coating the system was placed in an incubator at 37 °C. For HUVEC culturing the chambers were coated with human Fibronectin (663, YO Proteins AB) 100 µg/mL in cell culture medium for 20 minutes at room temperature. The cell culture chip was then flushed with cell culture medium for 30 minutes at a flow rate of 5.2 µL/min. Approximately 10 µL of cell suspension was loaded into wells of a PDMS loading chip (Figure 5). The cell density for HeLa cells, adipose-derived stem cells, PC12 cells, and HUVEC was 2 x 10^5 , 1 x 10^5, 5 x 10^5, and 1 x 10^6 per mL respectively. To introduce cells into the cell culture chip, the micropumps were programmed to run backwards at a flow rate of 5.2 µL/min for about 1 minute. This introduced cells into the chambers from the outlet side. The system was then placed in an incubator at 37 °C and 5% CO2. To allow cell attachment to the surface, the cells were left without flow for 2 hours. Following this, the cells were perfused at a flow rate of 500 nL/min for HeLa cells, ASC (Isolation, culture and banking of adipose stem cells was done according to protocols approved by the Regional Committee for Ethics in Medical Research for Southern Norway (approval S-06378a to P.C.)) and PC12 cells and 400 nL/min for HUVEC until completion of the experiment. For time-lapse microscopy the entire system was enclosed in an incubator (Incubator XL Dark S1, Carl Zeiss, Germany) at 37 °C when mounted in the microscope. To limit the influence of gas permeable materials on the liquid and thereby keep the pH of the culture medium at approximately 7.0-7.2, the entire system incubator was humidified with 8% CO2 enriched gas mixture (CO2 module S1, Carl Zeiss, Germany). 10X phase contrast time-lapse images of cell proliferation for HeLa cells, ASC, and PC12 cells were recorded every 12 hours by a Zeiss Axio Observer.Z1 microscope equipped with a Zeiss Axiocam MRm B/W camera. A scan of 2×7 images, all acquired with a z-stack of eleven slices (5 µm between each slice), were recorded for each chamber. For HUVEC the whole system was incubated in a conventional incubator at 37 °C and 5% CO2 and then moved to the microscope every day for imaging. 4X phase contrast images acquired by a Nikon Eclipse TS 100 equipped with a Nikon Digital Sight DS-2Mv.

PC12 cells were inserted into and cultured in MainSTREAM for 4 days before initiation of differentiation as described above. Medium reservoirs were changed to reservoirs with differentiation medium (DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 0.5 % v/v Horse serum (HS) (H1138, Sigma), 0.5 % v/v Foetal Bovine Serum (FBS) (F9665, Sigma), Heps 0.5 % v/v (H0887, Sigma), Nerve growth factor (NGF) 0.1 µg/mL, penicillin 100 U mL-1, and streptomycin 100 µg mL-1 (P4333, Sigma). Cell culture and differentiation of PC12 cells in MainSTREAM were continued for further three days and 10X phase contrast images recorded every six hours to follow differentiation.

In situ hybridization
An in situ hybridization (ISH) assay was performed by attaching the HistoFlex device to a MainSTREAM configuration. In brief, HistoFlex encompasses a patterned PDMS insert which upon sealing aligns microfluidic reaction chambers to tissue sections fixed on a microscope slide (Søe et al 2010). Integrated channels in the PDMS insert permit fluid delivery to and from reaction chambers. Temperature is controlled using peltier elements. In this report, HistoFlex’s fluidics were driven and controlled using MainSTREAM components: the described valve component, a micropump and interconnection components (Figure 8). HistoFlex is not part of the MainSTREAM platform and its interconnection features are not directly compatible with MainSTREAM components. As such, HistoFlex was connected to the MainSTREAM components previously listed through a merging chip (MC), and a variation of tubing connector (TC)(Figure 8). The MC merged the output of the eight channels; first into groups of 6 and 2 channels, and subsequently into a single channel (Figure 5b). The single channel was connected to the HistoFlex using the TC. Casting techniques are used to create an o-ring feature at the end of a 1.6 mm through channel found within a PDMS block housed in a polymeric bracket. The bracket was attached to the MC using 2 mm screws. 0.2/1.7 mm inner/outer diameter tubing (Bola/Bolender, Germany) was then press fit into the PDMS block. The other end of the tubing was inserted into a similar cavity in the HistoFlex PDMS insert, which connected to a reaction chamber. Waste from the reaction chamber was collected through tubing into a vial.

An ISH assay was performed for detection of microRNA-138 (miR-138) in formalin-fixed paraffin-embedded (FFPE), mouse brain tissue sections, by adapting the protocol and solutions presented by (Søe et al. 2010). Reagents were prepared in 0.2, 1.5 or 9 mL vials and placed in the vial rack (Figure 8). Vials, as depicted in Figure 8, were filled as follows: Channel 1 (C1) = PBS, C2 = prehybridization buffer (Søe et al 2010), C3 = hybridization buffer (40 nM miR-138 in 1xSSC, 4M urea and 1xDenhardt’s), C4 = 0.1 x SSC, C5 = blocking buffer, C6 = Cy3 - tyramide signal amplification solution, C7 = antiFITC antibody conjugated to a horse radish peroxidase (HRP) enzyme, C8 = PBS. Merging all channels of the MC at a single point could result in unwanted mixing of reagents at this single merging point. In particular, premature reactions between anti-FITC antibody coupled to a peroxidase enzyme (C7) and i) probe coupled to a fluorescein (FITC) label (C3), and i) tyramide signal amplification substrate (C6). For these reasons, the two point merging structure of the MC was used. To further avoid the aforementioned reactions, PBS(C8) was always flowed in between when switching reagents found from C1 to C6, and from C7 to C8. The MC was primed before connecting the HistoFlex to the microfluidic network. The MC was primed by sequentially filling each channel through pumping of the respective reagents past the channel merging area. After priming, the tubing was removed from the waste vial and connected to the HistoFlex.

Results

The results of the holistic approach to microfluidics are on two levels. Firstly, the engineering level which includes system assembly, pumps, valves and other components. Secondly, microfluidics systems were built to address three large groups of biological assays including genotyping, in situ hybridization and cell culture. Each of these required different components and system design. These examples are used to demonstrate and describe the versatility of the components. Pumps and valves were evaluated for robustness and performance parameters. Systems were generally evaluated for compatibility with intended assay type. In case of cell culturing systems, a detailed usability and robustness analysis was done on the system level using a novel cell culturing system described here and a previously reported system (Skafte-Pedersen et al. 2011).

System assembly principles

The MainSTREAM component library is summarized in Table 1. The principle used for creation of MainSTREAM microfluidic systems is the alternation of hard (rigid) and soft (compressible) components. Specifically, compression of the interconnection features of PDMS-based components against microfluidic elements such as microfluidic chips creates sealed microfluidic
networks. A sample assembly process of a system demonstrating this principle, inclusive of multi-channel pumping capability, is shown in Figure 6.

µFRs (Figure 1) contain aligned banks of 8 short tubes bookended with integrated interconnection features, which provide sufficient seal capabilities for most microfluidic applications (Sabourin, Snakenborg et al. 2009; Sabourin, Snakenborg et al. 2010). µFRs provide a simple flexible method for establishing chip-to-chip and chip-to-world interconnectivity and withstood system handling and moving operations (see below). Dead volumes are 2.5 and 1 µL respectively for Type I and II µFRs. A chip is exchanged by releasing the µFRs from chips by loosening screws, a new chip is inserted and the screws are then tightened to fasten the µFR. Dependent on chip design and number of µFRs attached to it, this usually takes less than one or two minutes. Complete system disassembly is thus not necessary to exchange a chip. The interconnections are robust and only seldom, leaks were observed from assembled systems despite having up to 96 interconnections (Figure 6, 12). Replacing the µFR from the pump only requires that the RB is removed and the ends of µFR are unfastened. The access of the parts from the top simplifies replacements of specific parts.

**Micropump performance**

The micropump is self-priming, and simultaneously pumps fluids through the Type I µFR’s eight channels bi-directionally (supplementary video 1). Average per channel displaced volumes per micropump rotation was approximately 0.7 µL (testing described in supplemental information). Heretofore, rotation refers to a 360° rotation. Channel-to-channel coefficient of variation for per channel displaced volumes ranged between 2.8 % and 8.7 % (Supplementary Information). Durability as compared to previously peristaltic pump (Skafte-Pedersen, Sabourin et al. 2009) was improved at minimum 30-fold. Testing (supplemental Information) indicated that following 63000 micropump rotations Type I µFRs were intact. Using the minimum observed per rotation channel displaced volume of 0.52 µL (supplemental information), 63000 rotations correspond to a minimum total displaced volume of 33 mL per channel. A 3-week microfluidic cell culture experiment running at 1 µL/min would consume 30 mL per channel. Additionally, given that testing was voluntarily stopped this is likely an underestimate of durability. The compact nature of the micropump allows its placement adjacent to microfluidic chips and yields compatibility with detection equipment (Figure 10d).

To achieve a given flow rate using the LEGO Mindstorms motors a procedure involving a) the use of a calibration chip (to determine per channel displaced fluid volumes per micropump rotation) and b) “STEP and WAIT” programming of LEGO® motors (supplemental Information) at powers allowing consistent turning of the micropumps is used. STEP and WAIT programs STEP the motor a fixed number of degrees and then WAIT a fixed time between steps. STEP and WAIT is enabled by the motors’ integrated rotation sensor, to within 1°, and simplified by the provided LEGO® LabView® interface. A sample STEP and WAIT procedure is provided in the supplemental information. Assuming an average per rotation per channel displaced volume of 0.7 µL, use of the LEGO® motors can provide flow rates from sub µL to 90 µL/min. Though per rotation micropump displaced volumes are constant from 25 to 50% motor power, flow rates do not linearly increase with power (data not shown).

**Valve performance**

Camshaft valve testing (supplemental information) showed that the valve could block channels against an upstream pump running at 100% motor power. With an open valve this corresponds to a flow rate 89 µL/min, well above that used for the majority of microfluidic applications. The pressure buildup between the micropump and valve was automatically relieved by backflow towards the micropump. Valve rotation between different positions occurred in less than 2 seconds. This time was dependent on the degrees of rotation required. Valve durability testing (supplemental information) was voluntarily stopped after 20000 rotations. Each rotation corresponded to an opening and closing of each of the µFR’s eight channels. After 20000 rotations, the µFR was not...
damaged and all eight channels were still being properly valved. Given that testing was voluntarily stopped this is likely an underestimate of durability. For typical biochemical assays (see discussion) this is sufficient for approximately 2000 complete assays.
<table>
<thead>
<tr>
<th>Component</th>
<th>Interfacing Characteristic</th>
<th>Description</th>
<th>Fig</th>
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<tbody>
<tr>
<td>Microfluidic Ribbons (uFRs) Type I</td>
<td>Soft</td>
<td>Simultaneous 8 channel through micropump and camshaft valve connectivity 16 self-aligning minimal dead volume interconnections 2.5 µL internal dead volume</td>
<td>1</td>
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<tr>
<td>Microfluidic Ribbons (uFRs) Type II</td>
<td>Soft</td>
<td>Simultaneous 8 channel through micropump and camshaft valve connectivity 16 self-aligning minimal dead volume interconnections 1.0 µL internal dead volume</td>
<td>1</td>
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<tr>
<td>Micropump</td>
<td>Soft</td>
<td>Bi-directional, self-priming, simultaneous pumping of 8 channels from nL to approx. 90 µL/min Low compliance, compact, portable, compatible with detection methods</td>
<td>2</td>
</tr>
<tr>
<td>Camshaft Valve</td>
<td>Soft</td>
<td>Modular, user defined valving patterns of 8 channels Compact, portable, compatible with detection methods Valves channels up to 89 µL/min</td>
<td>3</td>
</tr>
<tr>
<td>Microfluidic Chip</td>
<td>Hard</td>
<td>Must contain 10 hole MainSTREAM pattern to interface to soft elements</td>
<td>4</td>
</tr>
<tr>
<td>Well Arrays (WA)</td>
<td>Hard</td>
<td>Sample and waste reservoirs which eliminate tubing requirements Must contain 10 hole MainSTREAM pattern to interface to soft elements Can be pressurized for prevention of bubbles in MainSTREAM networks</td>
<td>5</td>
</tr>
<tr>
<td>Tubing Connector (TC)</td>
<td>Hard</td>
<td>Simultaneous connection of 8 sample vials to µFRs</td>
<td>5</td>
</tr>
<tr>
<td>Connector chip (CC)</td>
<td>Hard</td>
<td>Direct interfacing of µFRs to each other</td>
<td>4</td>
</tr>
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Genotyping using MainSTREAM system

We have previously described incorporation of DNA arrays within PMMA microfluidic chips (Sabourin, Petersen et al. 2010) to genotype patients for mutations in the beta-globin gene \((HBB)\) giving rise to beta-thalassemia. \(HBB\) mutations are prevalent in resource poor areas. A key requirement for diagnostics in such areas is to lower costs. One way to do so is increasing the tests and patients analyzed per device. Microfluidic lanes of our previous chip were addressed with a pipette and used 4.5 mm channel spacings. Reduction of this spacing would increase risk of cross-contamination. To avoid this problem the microfluidic DNA chip (Sabourin, Petersen et al. 2010) was modified with MainSTREAM’s standard 10 hole patterns and connected to two handcrank driven micropumps (Figure 7 and supplementary video 3).

![Figure 7 MainSTREAM ASH genotyping. (a) MainSTREAM hand crank-driven micropumps interfaced to PMMA genotyping chips contained 16 lanes. Representative system and chip shown with alternating lanes filled with red and blue dye to facilitate visualization. Each chip lane contained an ASH diagnostic microarray for genotyping HBB mutations. Reservoir chips (arrows), made from PC, are mounted to the top side of micropumps and contain wells in which samples and reagents can be introduced. Turning of the hand crank drives samples through the microfluidic chip. (b) ASH genotyping. The image shows microarray hybridization following processing with a hand crank driven MainSTREAM system. ASH microarrays contained Wild Type (WT) and Mutant Type (MT) probe spots for HBB mutation sites labelled above as I, II and III above. Hybridization of patient-derived target to only WT probes indicates the patient is homozygous unaffected. Similarly, hybridization to only MT probes indicates the patient is homozygous affected. Hybridization to both WT and MT probes indicates the patient is heterozygous for a mutation at a given site. In this case, the patient was heterozygous at mutation site I. Patient material was fluorescently labelled for detection purposes.

WA chips were attached directly to the pumps’ Type I μFRs. The WA’s design provided features guiding operators and more suited to introduction of solutions by pipette. Genotyping now occurred on chips featuring 16 lanes spaced at 2.25 mm (Figure 7). The PMMA chip contained a microarray of DNA probes (Supplementary Table S2) for genotyping mutations in the \(HBB\) gene. Patient DNA samples were hybridized to the DNA microarrays found within the microfluidic chip (Sabourin, Petersen et al. 2010) lanes by loading the samples in WA and turning the hand crank. By turning the hand cranks, sixteen samples were introduced nearly simultaneously into the PMMA chip (supplementary video 2) and four patients were genotyped at once using a multi-stringency washing protocol (supplementary Figure 5). Four out of four patients were correctly genotyped for three mutations in the HBB gene using this highly portable non-motorized approach (Figure 7).

In Situ Hybridization (ISH) using valve and pump components

ISH assays are an example of histological assays. These assays are used in pathology investigations and aim to detect the presence of various diagnostic markers, e.g. nucleic acid sequences, within preserved tissue sections. At present a lack of reproducibility in
histological assays is thought to limit their clinical relevance and potential (Blenkiron, Goldstein et al. 2007). Reproducibility issues stem from variability in processing steps such as sample pretreatment and hybridization conditions (Nitta, Kishimoto et al. 2003) (Hrabovszky and Petersen 2002). Additionally, the utility of ISH assays is limited as they are typically manually operated and take extended time periods, from a single to several days, to complete. For instance obtain staining of miR-138 (see below) in conventional ways requires incubation of the tissue slide with 8 different reagents and totally 18 incubation and washing steps (Soe, Okkels et al. 2011).

The HistoFlex device (Soe, Okkels et al. 2011) (Figure 8) has recently been described as a tool for generating high performing histological assays. The Histoflex encompasses a patterned PDMS insert which upon assembly, seals a microfluidic reaction chamber to a tissue section fixed on a microscope slide (Soe, Okkels et al. 2011). The HistoFlex enables real time monitoring of flow based assays over tissue sections and limits variability related to hybridization conditions. However, as a standalone device it still required manual and sequential introduction of reagents.

Fig. 8 In situ hybridization (ISH) assay. (a) System component outline and flow. (b) ISH setup. Reagents are stored in the vial rack prior to usage. Reagents flow from the vials through the micropump and towards the valve. They are routed through a merging chip (MC) and then flow into the HistoFlex. A program was written and stored on the LEGO® NXT controller which automated the assay by co-ordinating and controlling the motors used for micropump and valve operation. For demonstration purposes, micropump and camshaft valve channels have been filled with either water or coloured dye solutions (blue, green and red). The HistoFlex reaction chamber is filled with red dye. Excluding HistoFlex temperature control components, the system footprint is approximately 30 x 20 cm.

To automate reagent introduction and reduce ISH assay times the HistoFlex was interfaced to a MainSTREAM system (Figure 8). The reagents required for the ISH assay were placed in reagent vials which were connected to the micropump by a TC. The micropump was then connected to the camshaft valve using a CC. As the HistoFlex is not part of the MainSTREAM platform, its interconnection features are not directly compatible with MainSTREAM components. To solve this, a merging chip with the 10-hole pattern permitting connection to the camshaft valve was connected to the HistoFlex using a variation of TC (supplemental information). The LEGO® Mindstorms® motors and software interface were used to control the micropump, camshaft valve, and hence gate and automate the introduction of 8 different solutions into the HistoFlex. In brief, the
micropump was programmed to run at 89 \( \mu \text{L/min} \) (full speed) when filling or replacing reagents in the reaction chamber. Once the reaction chamber was filled with a reagent, the micropump was programmed to stop for static incubation, to deliver continuous flow at 41 \( \mu \text{L/min} \), or to introduce approximately 0.6 \( \mu \text{L} \) every 15 seconds for discontinuous incubation (supplemental information).

Using the system shown in Figure 8, an ISH assay was performed for detection of microRNA-138 (miR-138) in formalin-fixed paraffin-embedded mouse brain tissue sections by adapting the protocol previously described by Søe et al (Søe, Okkels et al. 2011). Results of the ISH assay are shown in Figure 9. Automation of the ISH assay using the MainSTREAM network resulted in a reduction of assay times to approximately 4 hours which is half the time as for conventional methods (Søe, Okkels et al. 2011) and, with the exception of initial sample vial fillings, eliminated the need for an operator. Use of the LEGO® motors and the provided user interface made co-ordination of micropump and camshaft operation a simple task. Use of the camshaft and micropump yielded a decrease in assay variability through increased reproducibility of both reagent flow rates and reaction times (Soe, Okkels et al. 2011).

![ISH for detection of microRNA-138 in mouse brain tissue sections. Fluorescent images show miR-138 in red (cy3) and nuclei in blue (DAPI). (a) Low magnification (2.5x) of hippocampus and adjacent cortex. High magnification (20x) images show cell specific detection in hippocampus (b), cortex (c) and Purkinje cells of the cerebellum (d).](image)

**Cell culture and differentiation using MainSTREAM systems**

Cell culture is a more demanding application of microfluidics. Sterile working conditions must be maintained throughout experiments and experimental protocols frequently require different workstations: e.g., LAF bench, incubator, microscope/observation tool. As such, portable and compact microfluidic systems for cell culture are beneficial. Additionally, in order to harness the advantages of microfluidic approaches systems should provide higher parallelization, throughput and multiplexing capabilities. To demonstrate that the MainSTREAM approach was suited to microfluidic cell culture, several different fluidic systems were built for cell culture and differentiation experiments. For these applications, MainSTREAM multi-micropump systems such as that shown in Figure 10 were used for the culture of HeLa, adipose-derived stem, and PC12 cells over a period of four days (Figure 11) in PMMA microfluidic chips. PMMA was used due to its biocompatibility (Stangegaard, Wang et al 2006; Stangegaard, Petronis et al, 2006))
Fig. 10 (a) Four micropump MainSTREAM system. This system is highly portable. Motors are easily disconnected and reattached enabling the system to be shuttled between workstations including (b) laminar air flow bench, (c) incubator and (d) microscope. This last panel demonstrates compability and ability to integrate MainSTREAM systems with detection equipment.

Fig. 11 Cell adhesion, proliferation, and differentiation. (a) HeLa cells, adipose-derived stem cells, and PC12 cells cultured in a PMMA chamber with a height of 500 µm and a volume of 3.37 µL. Cells perfused with a flow rate of 500 nL min\(^{-1}\). 10X Phase contrast time-lapse images acquired after 1, 2, 3, and 4 days of cell culturing. (b) Differentiation of PC12 cells initiated after 4 days of cell culture. 10X Phase contrast time-lapse images acquired after 0, 1, 2, and 3 days of differentiation. (c) HUVEC cultured in a PMMA chamber with a height of 250 µm and a volume of 3.75 µL. Cells perfused with a flow rate of 400 nL min\(^{-1}\). 4X Phase contrast time-lapse images acquired after 0, 1, 2, and 3 days of cell culturing.

For all cell types the procedures for system preparation, cell loading, cell culture, and imaging demanded that the system was moved between a LAF bench, an incubator,
and a microscope stage. In the LAF bench the system was assembled, sterilized, coated and loaded with cells. The whole system with motors and all was then either placed in a CO\textsubscript{2} incubator or fixed in the microscope for time-lapse imaging, demonstrating portability and compatibility with instrumentation of the system. Moving a system required that and four (one for each motor) electrical and then one tube pressurizing the whole system was disconnected. Disconnecting the system from the pressure and electrical sources took less than 30 seconds. To introduce cells into the microfluidic chips, the CLC was used (Figure 5 and 10) and the micropumps were programmed to run backwards. All of the cultured cell types adhered and proliferated well compared to conventional cell culture, showing that the systems supported cell culture studies (Figure 11). Pressurization of the system (Skafte-Pedersen, Sabourin et al. 2010) resulted in bubble-free operation.

PC12 cells respond to nerve growth factor (NGF) that stimulate them to differentiate and stop dividing, which makes them useful as a model for neuronal differentiation. After 4 days of cell culture of PC12 cells, differentiation was initiated by changing liquid reservoirs with normal cell culture medium to differentiation medium and differentiation was followed by time-lapse imaging every six hours for three days (Figure 11b). The growing network of axons demonstrates that the system is usable for PC12 cell differentiation and for on-stage time-lapse microscopy.

Human Umbilical Vein Endothelial Cells (HUVEC) were used as a cellular model for primary cell culture in a two micropump MainSTREAM system. This system was designed to be compatible with upright microscopy and resembled the system used for micropump characterization (supplemental information). As demonstrated in Figure 11C these primary cells adhered and proliferated, demonstrating the system also could be used for even more demanding cell populations. HUVEC cultured in commercially available iBIDI chips connected to commercial peristaltic pumps was morphologically comparable with HUVEC cultured in the MainSTREAM system (data not shown).

**Reliability evaluation**

In order to test the reliability and usability of systems made from MainSTREAM components, we registered types of failures encountered when using systems for cell culturing. Three systems were compared. 1) the most complex system shown here containing up to 32 inlets + 16 outlets to and from a single chip, 4 pumps and three WA (Figure 10), “Lego system”. 2) A slightly less complex system, “Procell”, presented earlier consisting of 24 inputs, 8 outputs, three pumps and large number of individual glass vial for liquid managements (Skafte-Pedersen et al 2011) and 3) a single chip driven by up to four syringe pumps. Reliability was evaluated for long term (2-3 weeks) of cell culture or until failure. An experiment with up to 4 chambers in the chip takes about 10h to start when using a simple syringe pump solution (Fig 12A). It was possible to start a 24 chambers experiment using Procell system in about 10h leading to a time per chamber of 30 minutes. Corresponding values for the Lego system was 16 chambers in 10h or 40 minutes per chamber. This is far more rapid than the 2.5 h it takes for a syringe pump system.

The failures of the systems were also investigated. Failures were typically of two types - leaks and bubble formations. The leaks was mostly due delamination of bonded chips and breaks in the uFR (data not shown). Chips driven without over pressurizing the entire system leads to bubble formation in the majority of the reaction chambers even after a few days of cell culturing (Fig 12C). In systems that where not put under over pressure, most of the system failures were observed during the culturing phase. Non pressurized system was deemed useless for further use in long term cell culturing due to the problems with bubble formation. However, non-pressurized system work acceptably for ISH assays. A large drop in bubble formation in chambers was observed when pressurising entire systems (Fig 12C). Component failures were then increased during the experimental setup phase (Fig 12C) instead of during the run of the experiment. Work with pressurized Procell systems was divided into an early learning phase (the 13 first
experiments) and a later usage phase (five last experiments). During the learning phase, there were many issues with system handling, leaks and broken components during the experiment setup, but also during the run. After the learning phase, the Procell system was repeatedly used for cell culturing experiment with >20 days of incubation (Fig 12A, five last experiments) and there were no issues during the run with bubbles or broken parts. However, there was at least one component failure in about 40% of the experiments during experimental setup phase (Fig 12C). Using the more complex Lego system (Fig 10) gave the same experience. No bubbles after about three week culture in any of the chambers, while three of four experiments gave problems in the experimental setup phase. The results indicate that the robustness of each component needs to be increased to limit issues during the setup phase. However, assembled systems are robust enough to support three weeks cell culturing including handling procedures to switch medium and microscopy at least twice a week and microscopy 3-4 times a week. In total, system were moved up to 20 times during a cell culturing experiment. It should be noted that no contaminations were observed using the systems based on Mainstream components.

![Graph](image)

**Figure 12.** Analysis of reliability and use parameter. The analysis concerned a simple syringes pump system with up to 4 reaction chambers, a Procell (Skafte-Pedersen et al 2011) system that where not pressurized, a Procell system that was put under over pressure and a Lego system (Fig 10A) containing some improvement in liquid management systems. A) Experiment setup time and cell culturing times. B) Average number of component in a system and number of component failures. C) % Chambers with bubbles/failures, and % system failures during the setup and during the run of the experiment.

**Discussion**

Our goal was the creation of a platform with wide applicability and whose components would provide i) simple and reliable sample-to-waste interconnections, ii) numerous fluidic inputs and outputs to chips, iii) pumping and iv) routing/valving capacity for these inputs, and a high degree of v) portability, vi) reconfigurability/flexibility in system design, vii) compatibility with detection equipment, and viii) usability. Finally, though not necessary for all applications, the approach should be biocompatible. The results and systems presented above shows that the MainSTREAM component library (Table 1) fulfils these criteria. As will be discussed below, the holistic approach taken here to
microfluidics makes the system usable enough to be used in profession settings even for demanding applications.

Modular microfluidic systems (www.epigem.co.uk; www.labsmith.com; www.thinxxs.com; Grodzinski, Yang et al. 2003; Pepper, Palsandram et al. 2007; Rhee and Burns 2008; Sun, Wang et al. 2008; Yuen 2008; Yuen, Bliss et al. 2009), are based on a standardized breadboard/baseplate into which everything (usually just chips) is plugged in. The underlying assumption in such an approach is that the breadboard’s spacing and other design criteria will satisfy the overwhelming majority of microfluidic application and assay requirements. MainSTREAM components are modular, reconfigurable and provide a method to establish many different fluidic networks suitable for many applications. As shown (Figures 6-10), each application requires widely different layouts of the systems, suggesting that a standard breadboard is appropriate for rapid prototyping of chip functionalities, e.g. by serial connection of standardized chips, but not for rapid prototyping of entire microfluidics systems. An overview of system (Table 2) that can be build by MainSTREAM components suggest that these components can be used in the majority of lab on a chip application.

The performance of the micropump is sufficient for most biological applications. The micropump’s performance is similar to commercial syringe pumps with respect to channel-to-channel variability (Skafte-Pedersen, Sabourin et al. 2009) and significant durability improvements make it not only generally more applicable but particularly more suited to extended experiments such as long-term cell culture than our previous report (Skafte-Pedersen, Sabourin et al. 2009). Comparisons to similar PDMS-based, mechanically actuated peristaltic pumps is difficult as these i) only present a single channel pump (Lim, Kim et al. 2003; Yobas, Tang et al. 2008; Du, Ye et al. 2009; Koch, Remcho et al. 2009) or do not provide comparable data (Gu, Zhu et al. 2004; Rhie and Higuchi 2008). The MainSTREAM micropump facilitates parallel operations on chip and limits dead volumes. This is especially useful for multiplexed experiments where conserving samples is desired. The flow rate range, from sub μL/min to near 90 μL/min, is appropriate for many applications. For instance, it is possible to pump at 33 nL/min per channel resulting in medium exchange rate of 150 minutes in a cell culture chamber that is 0.5 X 2 X 5 mm. The pulsatile flow by the peristaltic pumps is not affecting fluidics operation on chip like switch from one liquid to another (Skafte Pedersen, 2011). We have no evidence that the pulsatile flow has negative effects on cell or solid phase biochemical assays. The ability to drive the micropump at higher flow rates with the Mindstorms® motors is advantageous for assays like ISH (Figure 8 and 9). For the ISH protocol (supplemental information), delivery of 30 μL to exchange reagents as quickly as possible within the reaction chamber took 20 seconds. Mindstorms™ motors can also be programmed to deliver discreet volumes from the micropumps via the integrated rotation sensor and then wait a set time before delivering another similar or different volume. This is useful for one-time deliver of reagent packets or low perfusion schemes requiring even supply of reagents to benefit reaction kinetics. This is particularly useful for even cell loading into chambers. A rapid injection of a cell suspension limits effects of sedimentation since the time it takes to draw in a cell suspension is counted in seconds using the Lego cell culturing system (Fig 10).

The novel camshaft valve presented in this report is itself a reconfigurable component which co-ordinates opening and closing of all the μFR’s channels simultaneously. Unlike many other valves (Unger, Chou et al. 2000; Browne, Hitchcock et al. 2009; Hulme, Shevkoplyas et al. 2009; Pitchaimani, Sapp et al. 2009; Zhang, Lin et al. 2009; Simone, Perozziello et al. 2010) employing deflection of elastomeric membranes, the presented valve is located outside and apart from the microfluidic device it addresses (as is the peristaltic micropump). As compared to the use of screws (Weibel, Kruthof et al. 2005; Hulme, Shevkoplyas et al. 2009), the camshaft simplifies operation as it co-ordinates valving of multiple channels simultaneously. As an example, for 8 channel systems passing from an “all open” to an “all closed” configuration with screw-based valves requires manual adjustment of 8 individual screws. In contrast, the camshaft must only be rotated to a new position (Fig 8). Solenoids or piezoelectric driven tactile
pins could have been used to valve individual channels of the µFR rather than a DC motor controlled camshaft. However, doing so would add additional layers of actuation and control equipment. Additionally, solenoid use restricts system portability (Mark, Haeberle et al. 2010). Although not yet tested in cell culture experiments yet, there are no reason why the valve cannot be used for such application to provide large degree of flexibility to deliver cues to cell cultures (table 2).

MainSTREAM design concept enables construction of highly integrated systems that are best choice for experimental procedures requiring moving the system between laboratory functions as illustrated in the cell culture experiment where the system is moved between LAF benches, microscopes and incubators (Figure 10). The exemplified systems (Figure 6, 7, and 10) are integrating pumps (including motors) reagents and waste into a movable unit after disconnecting the power source. Disconnecting and moving MainSTREAM systems is a quick and robust procedure as three weeks cell culturing were successful despite moving systems between incubators, LAF benches and microscope stations up to about 20 times during the experiment (Fig 12). The reasons for the solution being robust are likely 1) the pump function as a valve when stopped, 2) no fluidic lines are disconnected, 3) disconnecting a electrical contact stops the pump and thereby stops any movement of the liquids, 4) disconnecting a pressure line does not perturb the fluidic lines because the pump function as a valve.

To provide professional scale analysis systems, systems must contain many inputs and outputs to the chip. Number of samples analysed on a chip is limited by pumping capacity, efficient methods to connect pumps to the chip and liquid management systems. Commercial producers of microfluidic systems and components such as ThinXXS and Dolomite provide miniaturized pumps and valving solutions. These solutions address only a single channel but occupy the footprint of MainSTREAM’s eight channel micropump or valve. Scaling the aforementioned commercial microfluidic solutions into multi-step serial systems such as the ISH (Figure 8) and 32-channel cell culture applications (Figure 10) would lead to fairly complex, encumbered setups that are likely not very portable or compact. The control equipment and systems required for systems built from the commercial systems would likely be equally much larger as compared to use of LEGO® modules we have demonstrated. The demonstrated integration of pumps, valves, reagent and waste management and motors results in systems (Figure 6 through 10) which are portable and scaled enough (up to 32 individual reaction sites) to be used in biological applications. MainSTREAM systems cannot likely match the multiplexing and throughput capabilities of the multi-layer elastomeric approaches including the mLSI (Unger, Chou et al. 2000; Gomez-Sjoberg, Leyrat et al. 2007; Taylor, Falconnet et al. 2009) and Braille displays (Gu, Zhu et al. 2004; Futai, Gu et al. 2006; Tung, Torisawa et al. 2007) methods. For example, a single camshaft valve cannot produce as many valve combinations as the mLSI and Braille methods. However, for the majority of applications the ability to generate this many combinations is not required (Hulme, Shevkoplyas et al. 2009). The simple valve sequence used in this report, which gates one of eight liquids at a time through a reaction chamber, suffices for many different biochemical and cell-based assays such as sandwich, competitive, and direct immunoassays, DNA, protein and small molecule analysis by microarrays, tissue section analysis (Figure 7 and 8), affinity-based sample preparation procedures and the stepwise addition of compounds required differentiate stem cells (Osakada, Ikeda et al. 2009).

Table 2 outlines the capacity of a four-motor system to support complex time dependent assay. Depending on number of variable parameters, 4, 8 or 16 independent factors can be distributed to 8, 2 or 1 reaction sites respectively. We are currently exploring the routing capacity of the valve component, which would allow eight independent factors to be added to up to 16 reaction sites. With respect to assays featuring parallelization, recent mLSI-based microfluidic high content methods employed 16 fluidic inputs (excluding inputs required for system control) (Gomez-Sjoberg, Leyrat et al. 2007; Taylor, Falconnet et al. 2009) that can be routed to different parallelized cell culture sites (Taylor, R. J., D. Falconnet, et al., 2009) leading to 256 unique experiments. The MainSTREAM system shown in Figure 10 has 32 fluidic inputs. It would be possible
to arrange the pumping capacity into similar combinatory fashion also reaching 256 different unique reaction sites (Table 2). This number of fluidic inputs pushes the limitations of microfluidic approaches away from fluidic concerns and towards those associated with data capture and analysis. For example, time-lapse data capture and analysis on 32 parallel cell cultures is a demanding task.

Recently, calls for standardized, cheap, interconnectable, and easy to build microfluidic systems that can connect together effortlessly have been made (Whitesides 2011). MainSTREAM meets these demands. The components presented in this report are low cost and built from affordable materials (e.g. the material cost for micropump and camshaft is approx. 10 USD each). MainSTREAM also uses a standard 10-hole pattern for fluidic I/O of 8 channels. Though overall chip dimensions are flexible with this approach, the chips presented in this report were single or double microscope slide size to facilitate use in standard processing, such as microarray spotters, and detection equipment (Sabourin, Petersen et al. 2010). Spacing of µFR channels was set at 1536 well plate standards (2.25 mm) in order to simplify future coupling to automated fluidic handling machines. This would permit highly dynamic addition and subtraction of compounds and agents from test systems and would leverage many of the same advantages described by others (Yu, Alexander et al. 2007; Meyvantsson, Warrick et al. 2008; Puccinelli, Su et al. 2010).
Table 2. Applications to up to four motor systems:

<table>
<thead>
<tr>
<th>Config.</th>
<th>Configuration</th>
<th>Suggested/supported applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 pump lines</td>
<td></td>
<td>• Cell cultures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Homogeneous enzymatic/binding reactions e.g. PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Heterogeneous reactions e.g. hybridizations</td>
</tr>
<tr>
<td>32 pump lines</td>
<td>P1,1, P2,1, P3,1, P4,1, P1,8, P2,8, P3,8, P4,8</td>
<td>• Programming cell responses over time by creating time and concentration dependent gradients of three soluble factors (one is a null buffer).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Optimize enzymatic/binding reactions as a function of three reagents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Four steps heterogeneous assay e.g. hybridization, washing and colorimetric detection of microarrays</td>
</tr>
<tr>
<td>16 pump lines + 16 gating</td>
<td>P1,1, P1,2, P1,3, P1,4, P1,5, P1,6, P1,7, P1,8</td>
<td>• Eight reagent solid phase assay such as in situ hybridization, immunochemistry</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Complex cell programming</td>
</tr>
<tr>
<td>16 pump lines + 16 gating</td>
<td>P1,1, P1,2, P1,3, P1,4, P1,5, P1,6, P1,7, P1,8</td>
<td>• 16 reagent solid phase assay such as in situ hybridization, immunochemistry</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Complex cell programming</td>
</tr>
</tbody>
</table>
Conclusion

The component library of the MainSTREAM platform provides interconnection, sample-to-waste liquid management, as well as pumping and valving components which are flexible, reconfigurable, modular, scalable, and provide a simplified method to build microfluidic systems. Accessible and easily implemented methods of controlling and automating fluidic networks were also described. Microfluidic systems built from MainSTREAM components are compact, portable, user friendly, compatible with detection instruments and laboratory workflows and can interface to auxiliary components. The varied applications demonstrate for the platform illustrate that the approach can encourage broader adoption of microfluidics as it yields the benefits of parallel and multiplexed experiments, scalable fluidic actuation and routing and simplified sample-to-waste connectivity and management.

Acknowledgements

This work was supported by Grant No. 2106-08-0018 “ProCell” and Grant No. 2106-05-0047 “BioXTAS”, under the Programme Commission on Strategic Growth Technologies, the Danish Agency for Science, Technology and Innovation, and Grant agreement no. NMP4-SL-2008-214706 “EXCELL” under the European Community Framework Programme 7. The adipocyte stem cells were a kind gift of Prof. Philippe Collas, University of Oslo.


Stangegaard, M. Petronis, S et al., A Biocompatible Micro Cell Culture Chamber (µCCC) for the Culturing and On-line Monitoring of Eukaryote Cells, Lab. Chip, 2006, 6, 1045 - 1051


**Supplemental Information**

**Tubing Connectors (TCs)**

For applications requiring interfacing to sample vials a tubing connector (TC) (Figure S1) can be used. TCs can connect both Type I or II uFRs to sample or waste vials using 1.6 mm outer diameter (OD) / 0.2 mm inner diameter (ID) PTFE tubing (Bola/Bohlender, Germany). TCs are made from two PC pieces (Figure S1a). Both pieces measure 30 mm (l) x 5 mm (w) x 5 mm (h). The first piece aligns eight lengths of PTFE tubing via 1.6 mm through holes and contains a custom-made PDMS gasket insert. The PDMS insert contains 1.6 mm channels, each ending with a 1.0 mm wide and 0.5 mm high O-ring feature (Figure S1b). The PDMS insert is manufactured similarly to the method described in our previous report describing PDMS-based interconnection blocks which feature aligned O-ring banks [1]. In this case, 1.6 mm PTFE tubing was used during the casting process to create the 1.6 mm channels within the gasket. The second PC piece has eight through holes which transition from 1.6 mm to 0.8 mm diameter. These correspond to the PTFE and hemi-spherical ball joint feature diameters respectively. Alignment and compression by screws results in a sealed interconnection between TC’s PDMS gasket and Type I µFR (Figure S1c).
Fig. S1 Tubing Connectors (TCs). (a) TCs are made from two polymeric chips. (i) A polycarbonate (PC) shell houses a custom-made PDMS gasket insert (dashed box). The PDMS gasket contains 1.6 mm diameter channels. At the end of each of these channels a 0.5 mm wide O-ring protrudes by 0.5 mm. (ii) A PC chip with throughholes which transition from 1.6 and 0.8 mm diameters. (b) PTFE tubing of 1.6 mm OD is passed through the chip containing the PDMS gasket. Each protruding PTFE tube is thus collared by an O-ring feature (indicated by arrow). (c) Using 2 mm screws the TC is interfaced to one end of a Type I µFR. Dashed boxes correspond to individual components. The upper dashed box is the bracket of the Type I µFR. Parts (i) and (ii) are aligned such that the tubings enter the 1.6 mm portion of part (ii). Part (ii) is also aligned to the bracket of the Type I µFR. The Type I µFR ball joint features thus align with the 0.8 mm channel portion of part (ii). With compressive force, the gasket of part (i) forms a seal between part (i) and (ii) and the ball joint features form a seal between the Type I µFR and the part (ii) of the TC. The lack of a seal between the 1.6 mm PTFE tubing and the 1.6 mm diameter channels in part (ii) is expected.

**Micropump Characterization: Displaced Volume and Durability Testing**

The microfluidic chip and MainSTREAM configuration used for per channel displaced volumes and durability testing is shown in Figure S2.

Fig. S2 Experimental setup for micropump displaced volume and durability testing. (a) Setup. A microfluidic chip (MC) with 16 fluidic channels with cross-sections of 1 mm x 1 mm is connected to two micropumps. LEGO® motors are not shown. (b) Motors were first run to introduce fluids into each channel. A “before” picture was then taken. The motors were then activated for 25 rotations. An “after” picture was taken at this point. Using the “before” and “after” pictures the average amount of fluid displaced per pump rotation could be calculated. This was repeated for different LEGO® motor power settings and was the procedure used for durability testing.

Two micropumps were tested at different LEGO® motor power settings to verify that per channel displaced volumes per pump rotation did not change with different motor powers. Micropumps were tested between 25 and 50 % motor power. These values were selected since at 20% the micropump did not always consistently turn over, and at 50% flow rates exceeded what we gauged to be typical of microfluidic assays (i.e. well above 10 µL/min). Increased motor power did not result in changes to per channel average displaced volumes per pump rotation (Table S1). Average displaced volumes per channel
were different between the two micropumps tested. We estimate that manufacture of the microfluidic calibration chip used to measure displaced volumes may impart a channel-to-channel variability of approximately 1%.

Table S1: Per channel displaced volumes per micropump rotation at different LEGO® servo motor motor powers

<table>
<thead>
<tr>
<th>MicroPump</th>
<th>Power Setting (%)</th>
<th>Average Displaced Volume (µL)</th>
<th>Maximum Displaced Volume (µL)</th>
<th>Minimum Displaced Volume (µL)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>0.71</td>
<td>0.76</td>
<td>0.67</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.73</td>
<td>0.78</td>
<td>0.69</td>
<td>4.8</td>
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<tr>
<td></td>
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<td>0.70</td>
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</tr>
<tr>
<td></td>
<td>40</td>
<td>0.74</td>
<td>0.78</td>
<td>0.70</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.73</td>
<td>0.76</td>
<td>0.70</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.74</td>
<td>0.78</td>
<td>0.68</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.62</td>
<td>0.66</td>
<td>0.60</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.64</td>
<td>0.69</td>
<td>0.60</td>
<td>3.9</td>
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<td>35</td>
<td>0.64</td>
<td>0.67</td>
<td>0.60</td>
<td>3.2</td>
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<tr>
<td></td>
<td>40</td>
<td>0.64</td>
<td>0.68</td>
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<td>45</td>
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<td>0.68</td>
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<td></td>
<td>50</td>
<td>0.65</td>
<td>0.68</td>
<td>0.62</td>
<td>2.8</td>
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</table>

Durability and lifetime testing results are shown in Figure S3. Testing was completed using a motor power setting of 30%. A larger disparity in displaced volumes between micropumps is initially observed (prior to 23000 rotations), as compared to the difference between micropumps noted at 30% power in Table S1.

At 23000 rotations, the RBs of the micropumps being tested were switched. At this point the difference between micropump displaced volumes collapses (Figure S3). This indicates that attachment of the RB to a micropump can result in differences in average displaced volumes. When testing was deliberately stopped following 63000 rotations, all 16 channels of the two Type II µFRs used in the micropumps were functional and did not show signs of damage.

**Step and Wait Micropump Flow Programming**

As provided the LEGO® Labview interface does not permit selection or entry of flow rates. To achieve a given flow rate a procedure using “STEP and WAIT” programming of
LEGO® motors at powers allowing consistent turning of the micropumps (approximately 30% or higher) was used. *STEP and WAIT* programs *STEP* the motor a fixed number of degrees and then *WAIT* a fixed time between steps. *STEP and WAIT* is enabled by the motors’ integrated optical controllers and simplified by the provided LEGO® LabView® interface. Knowing the time required to complete a series of *STEP and WAIT* operations, an average flow rate can be determined. The following example is provided for demonstrative purposes:

A. Displaced volumes were first determined per micropump using a calibration chip (Figure S1). A displaced volume of 0.7 µL per pump rotation is assumed.

B. *STEP and WAIT* is set up such that the LEGO® motor advances in 5° steps and waits 0.05 seconds between steps.

C. The user programs the LEGO® motor to repeat the *STEP and WAIT* procedure 720 times, for a total of 3600° degrees of rotation, or 10 rotations. From A, the displaced volume per rotation is 0.7 µL and as such the total displaced volume will be 7.0 µL.

D. The program is started and the time required to complete the 10 rotations is recorded.

E. Dividing the volume delivered, 7.0 µL from C, and the time recorded to complete the test from D, an average flow rate can be determined.

An example *STEP and WAIT* programming block in the LabView interface provided with LEGO® Mindstorms® shown in Figure S4.

![Fig. S4 STEP and WAIT programming loop. The LabView® interface provided with Mindstorms® is shown. A STEP and WAIT program can be constructed using three buttons (highlighted boxes) found within the column on the left-hand side: MOVE (meshed gears), WAIT (hourglass) and LOOP (circular arrows). Once added to the programming environment, (gridded area) parameters for each function can be set in the MOVE, WAIT and LOOP windows shown at the bottom of the figure. In this example, the MOVE function is set to activate through a 5° step at 30% power (red box). The WAIT function requests a 0.05 second wait (blue box) until the next operation. The LOOP function repeats the MOVE and WAIT functions a total of 720 times (green box), or a total of 720 x 5° = 3600° or 10 rotations. As such, 10 pump rotations are completed in 5° steps.](image-url)
Camshaft Valve Durability Testing

To assess durability of the valve, in particular the PDMS µFR, the valve was placed in a MainSTREAM-based (Sabourin, Skafte-Pedersen et al. 2010) microfluidic system shown in Figure S5. The LEGO® Mindstorms® kit and software interface was used to control and co-ordinate both valve and micropump operation during durability testing.

Visual inspection and use of a microfluidic chip (Figure S5) with eight individual lanes was used to assess durability. Each channel of the chip connected to a single channel of the valve’s µFR. All eight channels of the microfluidic chip (Figure S5) were first primed by activating the pump and rotating the camshaft through a complete rotation thereby sequentially opening and closing each of the eight channels. During camshaft rotation, the pump was stopped. For durability testing, the camshaft was first rotated one rotation clockwise and then one rotation counter-clockwise. This was repeated through completion of nine valve rotations. The tenth valve rotation was completed stepwise in order to sequentially test each µFR channel. Channels were tested by running the micropump at 69 µL/min and verifying whether liquid flowed properly according to the camshaft’s valving pattern. For the camshaft shown in this report, this meant that i) flow should only be observed through one of the eight channels of the µFR at a time and, ii) that leaks resulting from PDMS wear by the camshaft should not be observed elsewhere. In a given valve position when the micropump was activated, proper valving was indicated by hanging drop formation only at the microfluidic chip channel exit corresponding to the µFR channel the camshaft set as open. µFR channels were evaluated in this manner after completion of 10, 20, 30, 40, 50, 100, 200, 500, 2,000 and 20,000 valve rotations.

![Fig. S5 Camshaft valve durability testing. A MainSTREAM micropump (left) connected to sample vials (not shown, off to left) by a TC (left) delivers fluids through the camshaft valve (center) and to a microfluidic chip (right). The eight individual lanes of the microfluidic chip permitted assessment of valve functionality. Automation of durability testing was accomplished by programming LEGO® Mindstorms® motors. The motor controlling the valve is not shown.](image)

Genotyping in MainSTREAM

Table S2: Probes used for ASH genotyping

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Probe Sequence (5’ to 3’)</th>
<th>Type of Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 17WT</td>
<td>CCCCCCCCCCTTTTTTTTTTCCCTGTTGGGGCAAGGTG</td>
<td>A &gt; T</td>
</tr>
<tr>
<td>CD 17MT</td>
<td>CCCCCCCCCCTTTTTTTTTTCCCTGTTGGGGCTAGGTGA</td>
<td>A &gt; T</td>
</tr>
</tbody>
</table>
All signals were analyzed with GenePix Pro 6.1 (Molecular Devices, Sunnyvale, CA, USA) and analyzed according to the multi-salinity gradient washing method, which allow for the use of probes with different washing buffer optima in the same microarray (Poulsen, Soe et al. 2008). For each chamber and salinity condition used to genotype a patient, a normalized ratio for each probe pair was calculated as the signal from the wild-type probe (S_{WT}) divided by the sum of the signals from the wild-type and mutant probes (S_{MT}).

$$ \text{RATIO} = \frac{S_{WT}}{S_{WT} + S_{MT}} $$

For an individual with no mutation, homozygous wildtype (WT) at a given mutagenic site, at an appropriate stringency during the post-hybridization washing step the normalized ratio should approach an ideal value of 1.0. Likewise, the ideal ratio for an individual who is heterozygote (HZ) at a given mutagenic site, with one wild-type and one mutant allele, should approach 0.5. Finally, the ideal ratio of a homozygous mutated (MT) individual should approach zero. A more comprehensive discussion of the multi-salinity gradient method is provided elsewhere [2]. An example of the analysis of this genotyping approach is described in Figure S6.

Table S3: Reagents used for ISH Assay

<table>
<thead>
<tr>
<th>Step</th>
<th>Channel</th>
<th>Reagent</th>
<th>Composition</th>
<th>Flow</th>
<th>Time (min)</th>
<th>Temp. (°C)</th>
</tr>
</thead>
</table>

Fig. S6 Multi-salinity gradient washing genotyping in MainSTREAM. In this figure analysis of a patient for the mutation site referred to as IVS I+5 is shown (Table S2). As can be seen, when using 2.0 X SSC discrimination between MT and WT probes is not sufficient in order to confidently and correctly genotype the patient as being WT at this mutation site as the ratio lies between the ideal values for WT and HZ individuals. Using wash buffers of increasing stringency results in correct identification of the individual’s genotype, WT, at the IVS I+5 position as the ratio approaches the ideal value of 1.0.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Step</th>
<th>Buffer/Reagent</th>
<th>Continuous</th>
<th>Discontinuous</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C1</td>
<td>Washing buffer</td>
<td>PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C2</td>
<td>Prehybridization buffer</td>
<td>5xSSC, 4M urea, 1xDenhardt’s, 500 mg/mL yeast RNA</td>
<td>Continuous</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Discontinuous</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>C3</td>
<td>Hybridization buffer</td>
<td>40 nM FITC labeled probe, 1xSSC, 4M urea and 1xDenhardt’s 0.1 x SSC</td>
<td>Discontinuous</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>C4</td>
<td>Stringency washing buffer</td>
<td>Continuous</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C1</td>
<td>Washing buffer</td>
<td>Continuous</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C5</td>
<td>Blocking buffer</td>
<td>0.1M Tris pH 7.5, 0.15M NaCl, 10% fetal bovine serum (FBS)</td>
<td>Discontinuous</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>C1</td>
<td>Washing buffer</td>
<td>Antibody-enzyme Cy3 - TSA solution</td>
<td>Continuous</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>C7</td>
<td>Antibody-enzyme</td>
<td>Cy3 - TSA solution</td>
<td>Discontinuous</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>C8</td>
<td>Washing buffer</td>
<td>Continuous</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>C6</td>
<td>Amplification reagent</td>
<td>Continuous</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>C1</td>
<td>Washing buffer</td>
<td>Continuous</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>


Appendix 5

Conference proceeding 1

Mette Hemmingsen, Peder Skafte-Pedersen, David Sabourin, Rasmus Find Andersen, Anita L. Sørensen, Philippe Collas, and Martin Dufva: **Perfusion cell culture reveals a paracrine or autocrine signaling pathway involved in adipose-derived stem cell differentiation into adipocytes.** Published in Proceedings of the Fifteenth International Conference on Miniaturized Systems for Chemistry and Life Sciences, pp. 834-836, 2011.
PERFUSION CELL CULTURE REVEALS A PARACRINE OR AUTOCRINE SIGNALLING PATHWAY INVOLVED IN ADIPOSE-DERIVED STEM CELL DIFFERENTIATION INTO ADIPOCYTES

Mette Hemmingsen¹, Peder Skafte-Pedersen¹, David Sabourin¹, Rasmus Find Andersen¹, Anita L. Sørensen², Philippe Collas², and Martin Dufva¹*

¹ Department of Micro- and Nanotechnology, Technical University of Denmark, DENMARK and ²Institute of Basic Medical Sciences, Stem Cell Epigenetics Laboratory, University of Oslo, NORWAY

ABSTRACT

Microfluidic cell culture has advantages compared to conventional static cell culture regarding spatial and temporal control of near-cell environment. Differentiation of adipose-derived stem cells (ASC) into adipocytes was studied under microfluidic perfusion culture conditions. Constant removal of cell released components from a culture had large negative impact on differentiation. Adding supernatant from cells, undergoing differentiation into adipocytes in static culture, to the perfusion medium restored efficient differentiation. The results suggest that a paracrine or autocrine signalling pathway is involved in differentiation of ASC into adipocytes.

KEYWORDS: Perfusion cell culture, Adipose-derived stem cells, Differentiation, Paracrine, Autocrine

INTRODUCTION

Microfluidic cell culture is an emerging field in stem cell research [1] owing to advantages compared to conventional static cell culture. By having constant perfusion of medium over the cell culture, the near-cell environment can be precisely controlled with respect to for example chemical stimuli via the cell culture medium. Constant perfusion will remove secreted factors from the cells and thereby disturb cell to cell signalling, which normally would be considered as a drawback as compared with static cell cultures.

ASC are an abundant source of adult stem cells with multipotent differentiation properties [2], no ethical or immunoreactive considerations, and potential for tissue regeneration and engineering. Differentiation of ASC into adipocytes is regulated by a network of transcription factors induced by insulin, glucocorticoids, agents elevating cAMP, and serum factors [3]. However, little is known about the role of paracrine or autocrine signalling in adipogenesis, and conventional static culture systems have been unsuitable for such investigations. In this study, we use a previously described microfluidic cell culture system [4] to disrupt paracrine and autocrine signalling pathways during stem cell differentiation. The possibility of disrupting signalling pathways is necessary for identification of cell released factors.

EXPERIMENTAL

The microfluidic system (Fig. 1a) [4] consists of three miniaturized eight-channel peristaltic pumps, liquid reservoirs and an exchangeable microfluidic chip with up to 24 culture chambers (Fig. 1b). Glass vials, lids, and PTFE tubing (BOLA 1810-01, Bohlender GmbH) were sterilized by autoclaving before assembling the system. Tubes connecting the liquid reservoirs to the pumps and the cell culture chip were filled separately with Milli-Q water to remove bubbles, before clicking on the cell culture chip to the system base plate. Inlet and outlet reservoirs were coupled with PTFE tubing (BOLA 1810-10) and supplied with sterile filtered air added 5% CO₂. To avoid formation of bubbles, a pressure of 0.3 bar was put on the flow system during the whole system preparation and cell culture period, only interrupted when for instance changing liquid reservoirs.

Figure 1: Microfluidic system for culture and functional studies of cells. a) The platform assembled with chip, 24 inlet and four outlet reservoirs. b) PMMA chip containing 24 parallel chambers with a height of 500 µm and a volume of 3.2 µL.
Prior to cell culture, the flow system was sterilized with 0.5 M NaOH for 20 min at a flow rate of 5.2 µL/min. NaOH was removed by washing with sterile water (W3500, Sigma) for 30 min at a flow rate of 5.2 µL/min. The surfaces of the cell culture chambers were coated with collagen (40 µg/ml in sterile water, C3867, Sigma) at 37°C at a flow rate of 5.2 µL/min for 15 min followed by 195 nL/min for 45 min. Finally, the flow system was flushed with medium (DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 10% v/v newborn calf serum (N4762, Sigma), penicillin 100 U/mL, and streptomycin 100 µg/mL (P4333, Sigma)) for 30 min at a flow rate of 5.2 µL/min before cell loading. Uniform cell density over the entire culture chamber was obtained by loading the cells in culture medium supplemented 60% serum. To allow cell attachment, cells were perfused with medium supplemented 10% serum for 4 h at a flow rate of 33 nL/min at 37°C. After this attachment phase, cells were perfused at a flow rate of either 33, 125, or 500 nL/min at 37°C until completion of the experiment. Differentiation was induced after 3 days of culture at a cell confluence of approximately 80-90%. The cells were perfused with either normal adipogenic differentiation medium (AM) (cell culture medium supplemented with IBMX (isobutylmethylxanthine) 0.5 mM (I5879, Sigma), dexamethasone 1 µM (D4902, Sigma), indomethacin 0.2 mM (17378, Sigma), and insulin 10 µg/mL (I9278, Sigma), or conditioned medium (CM) (a 1:1 mixture of medium from ASC undergoing adipogenic differentiation in static culture and AM with twice concentration of differentiation factors). Medium reservoirs were exchanged with fresh medium every third day.

Images of ASC differentiation were acquired at 10x phase contrast using a Zeiss Axio Observer.Z1 microscope connected to a Zeiss Axiocam MRm B/W camera. Quantification of lipid-filled droplets was carried out using ImageJ by marking areas of four or more pixels within a set range of gray-values and a circularization degree of 0.9-1.0, i.e. almost circular.

RESULTS AND DISCUSSION

Based on the assumption that constant flow conditions would disrupt a possible paracrine or autocrine signalling, we examined differentiation of ASC into adipocytes by perfusing ASC cultures with normal adipogenic medium at three different flow rates (33, 125, and 500 nL/min). Formation of lipid-filled droplets was used as an indicator of differentiation into adipocytes. Flow rates of 125 and 500 nL/min had a negative impact on differentiation compared to 33 nL/min (Fig. 2 upper panel and Fig 3). Flow rates of 33, 125, and 500 nL/min correspond to a resting time of medium in the cell culture chamber of 97, 26, and 6 min, respectively.

![Figure 2: Conditioned medium restored differentiation of ASC into adipocytes. ASC was induced to differentiate by perfusing the cells at three different flow rates (33, 125, and 500 nL/min) with either normal adipogenic differentiation medium (AM) or conditioned medium (CM) (a 1:1 mixture of medium from ASC undergoing adipogenic differentiation in static culture and adipogenic differentiation medium with twice concentration of differentiation factors). 10x phase contrast images of differentiating ASC acquired after 16 days of differentiation.](image-url)

To investigate whether the decreased formation of lipid-filled droplets was caused by removal of cell secreted signalling factors during differentiation, ASC were differentiated by perfusion with conditioned medium (AM added supernatant from
cells undergoing differentiation into adipocytes in static culture). Perfusion with CM increased the formation of lipid-filled droplets compared to ASC perfused with AM alone, as shown in Fig. 2 and Fig. 3. Importantly, the effect was dependent on flow rate. Almost no positive effect of perfusion with CM over AM was observed at 33 nL/min. By contrast, perfusion with CM resulted in up to two times more lipid-filled droplets than perfusion with AM for flow rates of 125 and 500 nL/min (Fig. 3b). This suggests an involvement of paracrine and/or autocrine signalling factors in differentiation of ASC into adipocytes. As control to perfusion with CM, cells were perfused with medium added twice the concentration of differentiation factors, but this resulted in cell death. Cells perfused with normal cell culture growth medium led to no differentiation at flow rates of 125 and 500 nL/min. However, some spontaneously differentiated cells were observed by perfusion with growth medium at 33 nL/min in the outlet end of the chamber (data not shown). The presence of differentiated cells only at 33 nL/min and in the outlet end of the chamber, where released factor concentration would be highest, further point to an involvement of paracrine and/or autocrine signalling factors in ASC differentiation into adipocytes.

**Figure 3:** Effect of conditioned medium (CM) on differentiation of ASC into adipocytes was flow rate dependent. a) Number of lipid-filled droplets over time from cell loading. Induction of differentiation was started at day 3. Number of lipid-filled droplets were measured using ImageJ by marking areas of four or more pixels within a set range of gray-values and a circularization degree of 0.9-1.0, i.e. almost circular. b) Number of lipid-filled droplets at perfusion with CM compared to perfusion with AM at flow rates of 33, 125 and 500 nL/min. Data shown at day 19, i.e. after 16 days of differentiation.

**CONCLUSION**

We have shown that perfusion culture at flow rates of 125 and 500 nL/min have a negative impact on differentiation of ASC into adipocytes compared to perfusion at 33 nL/min. Furthermore, differentiation is restored when perfusing with adipogenic conditioned medium. This demonstrates that ASC differentiation into adipocytes is dependent on a paracrine and/or autocrine signalling pathway. Thus, we have proven that our microfluidic perfusion culture system is suitable for stem cell differentiation. It constitutes a flexible tool to fine-tune stem cell differentiation conditions, as well as investigate signalling pathways controlling differentiation.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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Appendices

Appendix 6

Conference proceeding 2

1. Wajid Hassan Minhass, Paul Pop, Jan Madsen, Mette Hemmingsen, Peder Skafe-Pedersen, Martin Dufva: Cell Culture Microfluidic Biochips: Experimental Throughput Maximization, 5th International Conference on Bioinformatics and Biomedical Engineering, (iCBBE) , 2011.
System-Level Modeling and Simulation of the Cell Culture Microfluidic Biochip ProCell

Wajid Hassan Minhass†, Paul Pop‡, Jan Madsen‡, Mette Hemmingsen‡, Martin Dufva‡
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‡Department of Micro- and Nanotechnology
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Abstract—Microfluidic biochips offer a promising alternative to a conventional biochemical laboratory. There are two technologies for the microfluidic biochips: droplet-based and flow-based. In this paper we are interested in flow-based microfluidic biochips, where the liquid flows continuously through pre-defined micro-channels using valves and pumps. We present an approach to the system-level modeling and simulation of a cell culture microfluidic biochip called ProCell, Programmable Cell Culture Chip. ProCell contains a cell culture chamber, which is envisioned to run 256 simultaneous experiments (viewed as a 16 x 16 matrix). We use an inverted fluorescence microscope to observe the experiments in real-time, allowing kinetic data analysis. We are able to automatically adjust the current experimental setup thus allowing, for the first time, conditional experiments. We propose a biochip architecture model and a comprehensive fault model that captures permanent faults occurring during chip operation. Using the proposed modeling and simulation framework, we perform an architectural level evaluation of two cell culture chamber implementations. A qualitative success metric is also proposed to evaluate chip performance in the presence of partial failures. Our results show that significant improvements in efficiency can be obtained using redundancy, providing improved chances to complete an experiment even in the presence of faults. This decreases the experiment repetition rate while increasing system productivity, saving time and reducing costs.

I. INTRODUCTION

During the last decade, microfluidic biochips have become an actively researched area [1]. By miniaturizing the macroscopic chemical and biological processes to a sub-millimeter scale, microfluidic systems enable the integration of various assays onto a single chip. The technology is referred to as lab-on-a-chip (LoC), as it offers a promising alternative to a conventional biochemical laboratory. In addition to the reduced geometrical dimensions, this miniaturization also results in reduced required reagent volumes, saving material costs. Moreover, because of the homogeneous reaction conditions at the micro level, microfluidic-based systems provide results of enhanced precision compared to the conventional biochemical analyzers [2].

There are two technologies for the microfluidic biochips: droplet-based [4] and flow-based [7]. Droplet-based biochips, also referred to as digital biochips, deal with discrete droplets on a two-dimensional electrode array and have so far been the focus of attention of the design automation community [5] [6]. In flow-based microfluidic biochips, the liquid flows continuously through pre-defined micro-channels using valves and pumps [7]. These flow-based biochips are fabricated using multilayer soft lithography techniques. Although the soft lithography technology has advanced faster than Moore’s law [9], the design methodology of these biochips is still based on the bottom-up customized approach involving multiple manual steps with minimum design automation. Each new chip is designed by creating and connecting the on-chip components based on the precise steps of a specific assay [14], thus the chips are termed as “Full Custom” biochips.

As the designs become larger and more complex, the current bottom-up full-custom design approach will not scale to the new designs, widening the gap between the manufacturing technology capability and the achieved design complexity. New top-down computer-aided design tools are required, which can offer the same level of support as the one taken for granted currently in the semiconductor industry [10].

As a first step, we need to model the biochemical applications separately from the architecture. Thies et al. [12] propose a software system called BioStream with two levels of abstraction for describing biological assays. The first layer consists of a BioStream library, which is used to write a program for describing the assay. The second abstraction layer, a fluidic instruction set architecture (ISA), interfaces the first level with the underlying biochip architecture. Their work serves as a first step for decoupling the architecture from the assays.

Amin et al. [11] propose a general-purpose microfluidic architecture called AquaCore and an instruction set (consisting of instructions, such as, <input id2, id1> and <mix id, time> where id1 is an input port, id2 is a reservoir and id is the mixer) to be implemented on this architecture. AquaCore can be programmed to run any assay, and is thus termed as programmable LoC (PLoC). Although such a general-purpose PLoC has the obvious advantage of fast
In this paper, we present an approach for the high level modeling and simulation of a cell culture microfluidic biochip called ProCell, Programmable Cell Culture Chip [15]. ProCell is envisioned to run up to 256 experiments simultaneously. We use an inverted fluorescence microscope to observe the experiments in real-time. Depending on the observed outcome of a particular experiment, we are able to automatically adjust the current experimental setup. Thus, for the first time to our knowledge, conditional experiments can be performed.

ProCell can be viewed as an ASPLoC as it can be used to implement a specific set of applications with reasonably high efficiency, e.g., in vitro drug testing, genomics, stem cell analysis, tissue engineering [15]. This would offer the possibility of breakthroughs in regenerative medicine, cancer research and developmental biology.

Biochips belong to the class of Micro-Electro-Mechanical Systems (MEMS) and exhibit many different types of faults. Modeling these faults and their impact is imperative in order to perform a realistic system analysis.

In this paper we propose a biochip architecture model and a comprehensive fault model. We model faults that cause partial failures in the chip during operation and propose a success metric $Q_s$, which provides a qualitative measure of the chip performance in the presence of partial failures. Using the proposed simulation framework, we provide an architectural-level comparison of two cell culture chamber implementations. Our results show that significant improvements in efficiency can be obtained using redundancy, providing improved chances to complete an experiment even in the presence of faults. This decreases the experiment repetition rate while increasing system productivity, saving time (one cell culture experiment can take many days to complete), and reducing costs, since the purified proteins and compounds used in the experiments are highly expensive.

The rest of the paper is organized as follows. Section II describes the details of the ProCell prototype operation. Section III.A presents the architecture model of the cell culture biochip and Section III.B gives details of the proposed fault model. Different redundancy schemes are discussed in Section III.C and Section III.D gives out the problem formulation. The simulation framework is presented in Section IV. Section V describes the experimental results and Section VI presents the conclusions.

II. PROCELL PROTOTYPE OPERATION

ProCell is a new microfluidic-based device (being developed at the Technical University of Denmark, DTU) for culturing and monitoring of living cells in real-time [15]. ProCell is aimed to be a powerful, user-programmable, completely self-contained open platform for performing high throughput conditional cell culture experiments.

The ProCell chip contains one large chamber with 16 inlets and 16 outlets. There are a total of 32 individual inlets carrying soluble compounds (e.g., growth factors), which connect to the chamber inputs through 16 independent two-way valves. ProCell cell culture chamber is a modified version of the well-characterized single experiment culture chamber developed at DTU [16]. Fig. 1 shows an example of a large chamber being divided into 8 virtual cell culture chambers using the laminar flow property of liquids at micro scales (each row can be viewed as a virtual chamber). Laminar flow is a distinguished category in the liquid flow classification [3]. Laminar flows are defined as the flow of fluids in parallel layers without any disruption between the layers. To carry out the experiment, first, valve D2 is opened and valve D1 is closed, allowing 8 different cell types to be pumped into the large chamber and since laminar flow conditions are applied eight corresponding stripes of cells are produced. After sedimentation, cells adhere to the bottom of the chip.
Then, valve D2 is closed and valve D1 is opened, allowing eight soluble compounds to perfuse over the cells in the perpendicular direction creating simultaneous experiments in the chip. Exposure of a cell colony to the soluble compound and monitoring its reaction is termed as an experiment. Since we have 8 cell colonies in each virtual chamber and there are 8 virtual chambers in total, thus we have 64 simultaneous experiments taking place in the chip. The fluid flow is managed by pumps that are controlled by electric motors.

A. Large Chamber vs Isolated Chambers

Fig. 1 shows a large chamber that is divided into 8 virtual chambers. Each chamber holds 8 cell colonies. Dividing the large chamber in such a way allows the in vitro conditions in the virtual chambers to resemble more closely to the natural in vivo conditions, since the cells are placed next to each other both horizontally as well as vertically.

A second choice of chamber architecture is to use 8 independent chambers physically isolated from each other. Just like the virtual chambers in the previous case, each isolated chamber also holds 8 cell colonies. The difference in this case is that the cells are placed next to each other only horizontally. Vertically, the isolated chambers have solid boundaries, i.e., each row (representing a chamber) is isolated from the rows next to it. Although such a scheme may have less resemblance with the natural conditions, it may prevent faults in one chamber, such as air bubbles, from advancing to the other chambers. We analyze the performance of both kinds of chamber implementations in this paper.

B. Conditional Experiments

The current ProCell prototype is able to run up to 64 experiments simultaneously. We use an inverted fluorescence microscope to observe the experiments in real-time, allowing kinetic data analysis. Depending on the observed intermediate outcome of a particular experiment through optical feedback, we are able to automatically adjust the current experimental setup thus creating conditional experiments.

ProCell uses the Zeiss AxioObserver Z1 inverted fluorescence microscope [19] for real time image capturing of the cell culture chamber over extended periods of time. The microscope is integrated with a Fujitsu high-end workstation CELSIUS R650. MCL-D2568 [20].

III. SYSTEM MODEL

A. Biochip Architecture Model

Fig. 2 shows the biochip architecture model. Each row in the model represents a chamber\(^1\). The architecture is represented by an 8x8 matrix (8 rows representing 8 chambers, where each chamber hosts 8 cell colonies). Each element of the 8x8 matrix hosts a cell colony, i.e., a separate experiment.

One primary step common to all cell culturing applications is the placement of cell colonies onto the chambers. In Fig. 2, 20 different cell colonies (C\(_1\) – C\(_{20}\)) are placed on the biochip. The cell colonies marked with different patterns represent the fault affected regions, see next section. Each cell culture experiment requires some sort of controls to ensure quality of compounds being inserted into the chamber (which may degrade over time since one experiment lasts for many days) and to guarantee that the conditions in the chamber (e.g., a certain compound concentration) are uniform (some cell colonies might consume the compound at a rate fast enough to deprive the cell colonies downstream of it, creating undesired non-uniform conditions). Positive and negative controls, provided by specific cell colonies depending on the application, are used for this purpose [17]. Cell colonies serving as positive controls are manipulated to give out positive signals (e.g., certain level of fluorescence) in the presence of desired conditions (e.g., desired concentration of the compound) and are placed as the last cell colony in the chamber as shown in Fig. 2 using notation C\(^*\).

Absence of a positive signal from a positive control means

\(^1\) In the rest of the paper, the word chamber will be used interchangeably for the virtual or isolated chamber (not for the large chamber).
that the chamber no longer holds the desired conditions and the experimental results of that chamber are no longer reliable. Cell colonies serving as negative controls are manipulated to give out negative signals (e.g., absence of a certain level of fluorescence) in the presence of desired conditions (e.g., desired quality/ concentration of the compound) and are placed as the first cell colony in the chamber as shown in Fig. 2 using notation C'. A negative control giving positive results also renders the chamber experimental results unreliable.

Different cell colonies can exhibit different properties in a chip and are thus placed at different locations in the chamber, based on the application. Some cell colonies (termed as Comm – communicator colonies) secrete compounds that are useful for the cell colonies placed downstream. Such colonies are placed close to the chamber inlets. It may also be possible that these secretions are instead harmful for the other cell colonies, in which case the cell colonies secreting these compounds are placed close to the outlets ensuring that they do not contaminate the chamber. Some cell colonies might be very rare or very expensive (and thus are considered high priority – HP) and would hence be placed at the location most suited to their requirements, making a compromise on the placement of other cell colonies (considered low priority – LP). HP and LP can also reflect the weight of a particular combination of cells and experimental conditions in a chamber, since some combinations may be more vital than others in order to test a certain hypothesis. The cell colony placement scheme (the order in which the colonies are placed) on a chamber is thus dependent on the properties of the cell colonies and the desired output of the experiments being run on the chip. Placement scheme can have an impact on the tolerance to faults, e.g., colonies placed close to the inlets are more susceptible to air bubbles than the ones placed close to the outlets. Based on the cell colony properties, the colonies placed on the chip in Fig. 2 are categorized as:

- **Negative Control**: C
- **Positive Control**: C'
- **Comm**: C2, C6, C12, C17
- **High Priority (HP)**: C1, C4, C8, C10, C15
- **Low Priority (LP)**: All others

### B. Fault Model

As discussed in Section II.B, there are 64 simultaneous experiments running on the chip, i.e., each cell colony represents a different experiment. Failure of an experiment caused by a fault is defined as a cell colony being unable to exhibit the reactive response, which it would exhibit otherwise under the applied conditions in the absence of that fault.

Different kind of faults can occur in a cell culture biochip. Some faults (e.g., mechanical pump breakdown, electronic motor failure) result in the failure of all the cell colonies in a chamber (complete chamber failure), whereas others (e.g., air bubbles enclosing one cell colony in the chamber thus modifying its conditions) may lead to the failure of some of the cell colonies in the chamber, i.e., a partial chamber failure. In this paper we model partial failures in the chip and propose a metric called Failure Index (FI) which is used to provide a qualitative success measure of the biochip performance in the presence of partial failures.

**TABLE 1**

<table>
<thead>
<tr>
<th>Fault Type</th>
<th>Possible Failure Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air bubbles</td>
<td>None or partial cell adhesion</td>
</tr>
<tr>
<td>Over-stressed cells</td>
<td>None or incorrect results which will not be reproduced upon repetition</td>
</tr>
<tr>
<td>Over-stressed cells</td>
<td>Complete or partial chamber failure</td>
</tr>
</tbody>
</table>

A quantitative measure of success of the biochip can be the total number of experiments run on the biochip without failure. However, the cell colonies placed on the biochip have different properties and failure of one cell colony may have a stronger impact compared to the other, e.g., failure of the negative control would result in the failure of the whole chamber. Thus a qualitative measure of success is required.

Table 1 presents the three most common faults that occur in cell culture biochips together with the corresponding errors and possible failure grades. Failure grade assignment is done to all 64 cell colonies on the biochip depending on the kind and/or number of cell colonies affected by the fault. Failure grade PL or PH is assigned if the fault occurs on a low priority or a high priority cell colony, respectively. If the fault occurs on a communicator colony or on all the negative/ positive controls available in a chamber, failure grade PH is assigned to all the colonies in the chamber marking a complete chamber failure. Failure grade CC is a chamber level failure grade marking complete chamber failure and FC is a chip level failure grade representing full chip failure.

Table 2 shows the value contributed by the fault type to the total Failure Index metric depending on the failure grade assigned to the fault. For example, a single PL grade failure contributes 1 failure point, whereas a high priority cell colony failure resulting in a PH grading contributes 2 failure points. Failure of the positive or negative controls or a communicator colony is ranked as a complete chamber failure equivalent to 16 failure points. Last column of Table 2 shows the range of PL failure index contribution for a chamber as {0-5, 16}. This represents that that if 5 cell colonies out of the 6 in a chamber (2 cell colonies are reserved for positive and negative controls) undergo partial failure, the failure index contribution is 5 points, whereas partial failure of all 6 colonies is ranked as a complete chamber failure equivalent to 16 failure points. Same ap-
plies for the failure index contribution of \(PH\) failure shown in the Table. Using different failure index contributions for different impacts allows qualitative evaluation of chip failure.

### TABLE 2

<table>
<thead>
<tr>
<th>Fault Impact</th>
<th>Failure Grade</th>
<th>Description</th>
<th>Failure Index Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial Chamber Failure</td>
<td>PL</td>
<td>Represents partial chamber failure, (l) in (PL) represents failure of low priority cells</td>
<td>(1/2\text{M} - l )</td>
</tr>
<tr>
<td></td>
<td>PH</td>
<td>Represents partial chamber failure, (H) in (PH) represents failure of high priority cells</td>
<td>(2\text{M} - l )</td>
</tr>
<tr>
<td>Complete Chamber Failure</td>
<td>CC</td>
<td>Represents complete failure of a chamber (e.g., loss of positive/negative control or the (Comm))</td>
<td>(2\text{M} )</td>
</tr>
<tr>
<td>Full Chip Failure</td>
<td>FC</td>
<td>Represents simultaneous complete failure of all chambers</td>
<td>(N^3 \times 2\text{M} )</td>
</tr>
</tbody>
</table>

The Failure Index of a chip is calculated by adding the failure index contributions \((FIC)\) of all chambers:

\[
FIC = \sum_{i=1}^{N} FIC_i
\]

where \(N\) is the number of chambers.

In order to calculate \(FIC\) of a chamber, first the failure grade of the fault is determined based on the cell colony type at which the fault occurs. Based on the failure grade, the \(FIC\) for chamber 1 can be calculated as,

\[
FIC_1 = \left[ \sum_{i=1}^{M} PH_i \right] + \left[ \sum_{i=1}^{M} PL_i \right]
\]

where \(M\) is the number of cell colonies in the chamber, \(PH\) represents the failure points contributed by any high priority cell failures in the chamber, and \(PL\) represents the failure points contributed by the low priority cell failures. The roof value for the first term is \(2\text{M}\) (16 in this case) which is applied in case the summation value exceeds \((M-l-1)\), where \(l\) is the number of cell colonies reserved for the positive and negative controls in the chamber. The roof value for the second term is also \(2\text{M}\) and is applied if the summation value exceeds \(2\times(M-l-1)\).

In Fig. 2, chamber 1 (marked with inlet A1) has an air bubble failure on \(C\). Loss of the negative control marks a complete chamber failure, thus \(FIC_1 = 16\). In chamber 6, cell colonies \(C_4\) and \(C_8\) fail to adhere to the chip and are flushed out. \(C_4\) and \(C_8\) are in categories HP and LP respectively. As per eq. 2, \(FIC_6\) is calculated as \((2+1) = 3\).

Similarly, \(FIC_2, FIC_3, FIC_4, \) and \(FIC_8\) are equal to 16. \(FIC_5\) and \(FIC_7\) are equal to 0. Failure Index of the whole chip is equal to sum of all \(FIC\) (1), i.e., 83 for this case. In the worst-case, \(FI\) is represented by a full chip failure \((FC\) in Table 2).

The success metric \(Q\) is thus calculated as,

\[
Q = \left[ \frac{N \times 2\text{M} - FI}{N \times 2\text{M}} \right] \times 100
\]

where \(N\) and \(M\) represent the number of chambers and the number of cell colonies in a chamber, respectively. For the current case, \(Q\) is \(128 - 83\) / \(128\) = 35.15%.

Note that chamber 6 and chamber 7 in Fig. 2 have the same placement scheme. Chamber 6 is affected with partial failures, whereas chamber 7 is fault free. The redundancy increases the success probability for that particular placement scheme. The redundancy schemes are discussed in the next section.

The faults listed here are permanent faults that occur during chip operation and are detected through optical feedback in real time. Based on the detection results, the experimental setup is adjusted to cater for the caused errors. Fault detection is not a part of the ProCell simulator since the faults are randomly generated and injected into the cell culture chamber during simulation. The simulator is used to architecturally compare two different cell culture chamber implementations.

### C. Redundancy Schemes

Different redundancy schemes can be introduced into the chambers in order to improve the chip reliability. Two types of redundancy schemes are proposed here: placement redundancy and control redundancy.

Placement redundancy is defined as placement of the same cell colonies in multiple chambers, i.e., the colony placement done in one chamber is repeated in multiple chambers. The number of chambers in which the same placement is repeated is termed as the redundancy level.

In this paper, two types of chamber implementations are under consideration as discussed in Section II.A. The primary difference between the two implementations, in terms of fault impact, is that in the virtual chambers (present in one large chamber) the air bubbles can easily spread from one chamber to the other.
However, this is not true for the isolated chambers since those are separated from each other using solid boundaries. Fig. 3 shows the placement redundancy schemes for the isolated chambers implementation. Fig. 4 shows the placement redundancy schemes for the virtual chambers. Maximum air bubble radius is set to three chambers. For redundancy levels 1 and 2 in Fig. 4, the redundant placements are made 2 chambers apart such that the air bubble affecting one chamber does not affect the other redundant chambers even in the worst-case.

Control redundancy is defined as placing multiple control colonies on a single chamber, e.g., instead of one negative control two negative controls can be placed on the chamber. Failure of the control marks the failure of the whole chamber. Thus, the placement of redundant controls can increase the success probability of the experiment.

D. Problem Formulation

The problem addressed can be formulated as follows. Given (1) a cell culture biochip architecture model $\mathcal{M}$ consisting of an $N \times M$ matrix representing the cell culture chamber, (2) a set of three different static cell colony placement schemes $\mathcal{P}$, (3) a fault model $\mathcal{F}$ together with the set of occurrence rates of different faults, and (4) a set of placement redundancy levels $\mathcal{R}$, the target is to perform an architectural level evaluation of two implementations: large chamber (with virtual chambers) vs isolated chambers. The evaluation provides qualitative measure of success $Q$ for each chamber implementation under the same conditions. The evaluation is made at different redundancy levels in both cell culture chamber implementations in order to determine the variation in $Q$.

The architectural evaluation involves mapping the placements $\mathcal{P}$ onto the chamber architecture model, conducting a property analysis of the placed cell colonies, randomly generating different faults according to the given occurrence rates (see Section V for details) and calculating the failure index $FI$ which is used to calculate $Q$. The performance evaluation is based on the properties of the architecture model, the fault model and the cell colonies at which these faults occur. The architectural comparison is performed independent of any specific application rendering the results equally applicable to all applications that can be executed on the chip.

IV. PROCELL SIMULATOR

The simulation framework is shown in Fig. 5. Cell chamber implementation choice, the placement scheme, fault rates and selected redundancy levels are given as an input to the simulator.

The first step in the simulator operation is to map the selected placement onto the selected chamber implementation. The chamber implementation is represented as a matrix model in the simulator. Different cell colonies have different properties and thus different priority levels as discussed in Section III.A. Based on the properties of these cell colonies, a new property matrix is generated next. This property matrix is utilized at the time of failure index evaluation since the same fault occurring on cell colonies with different properties can result in a different failure index contribution.

Next, using Monte Carlo Simulation (MCS), faults are randomly generated based on the chosen fault rates and evaluated to generate the Failure Index (FI) and the success metric $Q$. $FI$ calculation takes into account the fault model built in the simulator, the generated property matrix, selected chamber implementation and the chosen redundancy level. At the end of the MCS cycle (10,000 runs), the average percentage value of $Q$ is calculated and generated as an output.

V. EXPERIMENTAL RESULTS

Table 3 shows the results of the architectural comparison (as a measure of $Q$) between the two chamber implementations using varying fault rates. Only control redundancy is utilized for this comparison. The property based description of the three placement schemes is as follows:

$$P_1 = <—, \text{Comm, LP}, \text{LP, LP, LP, LP, +}>$$
$$P_2 = <—, \text{Comm, LP, Comm, LP, LP, HP, +}>$$
$$P_3 = <—, \text{Comm, LP, Comm, LP, LP, HP, +}>$$

As can be seen, $P_2$ and $P_3$ are identical, other than the fact that the $\text{Comm}$ of $P_2$ is replaced by an extra negative control in $P_3$, providing the control redundancy.

Different fault rates can be chosen for the three types of faults discussed in Table 1. Fault rates under which the simulation experiments were performed are given in the first column in Table 3. Since no statistics on the fault occurrence rates are available yet, we base our experiments on the limited observations made during the ProCell opera-
The experiments are carried out at different fault rates per experiment and the results are analyzed. Whenever more accurate statistics become available, those can easily be plugged into the simulator to perform architectural evaluation. The notation (10, 5, 5) in the first column of the Table means that the air bubble occurrence rate is 10%, rate of the faults resulting in cells not adhering to the chamber is 5% and the rate at which the cells become non-reactive is 5%, respectively.

### TABLE 3
RESULTS: PERFORMANCE COMPARISON

<table>
<thead>
<tr>
<th>Fault Rate</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10, 5, 5)</td>
<td>55.14</td>
<td>54.19</td>
<td>58.53</td>
</tr>
<tr>
<td>(20, 5, 5)</td>
<td>38.08</td>
<td>36.72</td>
<td>41.26</td>
</tr>
</tbody>
</table>

8 virtual chambers
(Max air bubble radius = 3 chambers)

<table>
<thead>
<tr>
<th>Fault Rate</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10, 5, 5)</td>
<td>44.25</td>
<td>43.15</td>
<td>48.02</td>
</tr>
<tr>
<td>(20, 5, 5)</td>
<td>23.30</td>
<td>21.58</td>
<td>23.66</td>
</tr>
</tbody>
</table>

8 virtual chambers
(Max air bubble radius = 5 chambers)

<table>
<thead>
<tr>
<th>Fault Rate</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10, 5, 5)</td>
<td>36.36</td>
<td>34.96</td>
<td>39.96</td>
</tr>
<tr>
<td>(20, 5, 5)</td>
<td>15.27</td>
<td>13.93</td>
<td>17.52</td>
</tr>
</tbody>
</table>

### TABLE 4
8 ISOLATED CHAMBERS: REDUNDANCY RESULTS

<table>
<thead>
<tr>
<th>Fault Rate</th>
<th>Redundancy</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10, 5, 5)</td>
<td>2</td>
<td>79.60</td>
<td>71.40</td>
<td>81.09</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>2</td>
<td>46.30</td>
<td>32.80</td>
<td>47.11</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>2</td>
<td>22.97</td>
<td>12.47</td>
<td>23.61</td>
</tr>
<tr>
<td>(40, 20, 20)</td>
<td>2</td>
<td>10.33</td>
<td>4.25</td>
<td>10.50</td>
</tr>
<tr>
<td>(10, 5, 5)</td>
<td>4</td>
<td>95.14</td>
<td>91.37</td>
<td>95.86</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>4</td>
<td>69.86</td>
<td>54.16</td>
<td>71.24</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>4</td>
<td>40.07</td>
<td>23.41</td>
<td>41.37</td>
</tr>
<tr>
<td>(40, 20, 20)</td>
<td>4</td>
<td>18.93</td>
<td>7.89</td>
<td>19.31</td>
</tr>
<tr>
<td>(50, 25, 25)</td>
<td>4</td>
<td>7.75</td>
<td>2.25</td>
<td>7.92</td>
</tr>
<tr>
<td>(10, 5, 5)</td>
<td>8</td>
<td>99.27</td>
<td>98.80</td>
<td>99.60</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>8</td>
<td>88.38</td>
<td>78.15</td>
<td>90.01</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>8</td>
<td>62.53</td>
<td>40.96</td>
<td>64.06</td>
</tr>
<tr>
<td>(40, 20, 20)</td>
<td>8</td>
<td>33.47</td>
<td>15.25</td>
<td>34.75</td>
</tr>
<tr>
<td>(50, 25, 25)</td>
<td>8</td>
<td>14.66</td>
<td>4.50</td>
<td>15.19</td>
</tr>
</tbody>
</table>

### TABLE 5
8 VIRTUAL CHAMBERS: REDUNDANCY RESULTS

<table>
<thead>
<tr>
<th>Fault Rate</th>
<th>Redundancy</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Max Air bubble radius = 3 chambers)</td>
<td>2</td>
<td>74.46</td>
<td>64.55</td>
<td>75.90</td>
</tr>
<tr>
<td>(10, 5, 5)</td>
<td>2</td>
<td>37.53</td>
<td>24.69</td>
<td>38.26</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>2</td>
<td>16.49</td>
<td>8.13</td>
<td>16.92</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>2</td>
<td>6.45</td>
<td>2.4</td>
<td>6.67</td>
</tr>
<tr>
<td>(40, 20, 20)</td>
<td>3</td>
<td>83.19</td>
<td>74.20</td>
<td>84.87</td>
</tr>
<tr>
<td>(10, 5, 5)</td>
<td>3</td>
<td>43.54</td>
<td>28.33</td>
<td>44.44</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>3</td>
<td>18.45</td>
<td>8.83</td>
<td>19.10</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>3</td>
<td>7.00</td>
<td>2.46</td>
<td>7.20</td>
</tr>
<tr>
<td>(40, 20, 20)</td>
<td>4</td>
<td>87.41</td>
<td>79.75</td>
<td>89.27</td>
</tr>
<tr>
<td>(10, 5, 5)</td>
<td>4</td>
<td>49.08</td>
<td>32.33</td>
<td>50.57</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>4</td>
<td>21.06</td>
<td>9.89</td>
<td>21.59</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>4</td>
<td>7.70</td>
<td>2.61</td>
<td>13.64</td>
</tr>
<tr>
<td>(10, 5, 5)</td>
<td>8</td>
<td>95.82</td>
<td>92.41</td>
<td>96.71</td>
</tr>
<tr>
<td>(20, 10, 10)</td>
<td>8</td>
<td>66.87</td>
<td>49.16</td>
<td>69.58</td>
</tr>
<tr>
<td>(30, 15, 15)</td>
<td>8</td>
<td>33.33</td>
<td>16.71</td>
<td>35.02</td>
</tr>
<tr>
<td>(40, 20, 20)</td>
<td>8</td>
<td>12.85</td>
<td>4.36</td>
<td>13.67</td>
</tr>
<tr>
<td>(50, 25, 25)</td>
<td>8</td>
<td>4.23</td>
<td>0.97</td>
<td>4.50</td>
</tr>
</tbody>
</table>

Since the air bubbles are inserted into the chamber through inlets, the cell colonies closer to the inlets are more susceptible to the air bubble faults than the ones close to the outlets. We assume a linear decrease of 1% in the occurrence rate when moving from the inlets to the outlets in the chamber, i.e., cell colony closest to the inlet has a rate of 10%, next one has a rate of 9%, and so on.

As shown in Table 3, the isolated chambers have a higher success rate $Q$ compared to the virtual chambers. As the fault rates increase, the gap in the success metric also widens. This is because in the virtual chambers the air bubbles can easily spread from one chamber to the other. However, this is not true for the isolated chambers since those are separated from each other using solid boundaries. Comparing results of $P_2$ and $P_3$, it can be seen that the control redundancy provides up to 5% increase in $Q$. Tables 4 and 5 show the variations in $Q$ as a result of placement redundancies for the same placement schemes.

As shown in Table 4, even a level-2 placement redundancy provides up to 24% increase in $Q (P_1 \text{ under } (10, 5, 5))$ compared to the results without placement redundancy in Table 3 for isolated chambers. $Q$ is calculated by using the average $Q$ calculated over an MCS cycle (10,000 runs). For every run, the higher value of $Q$ from the two redundant chambers (for level-2) is considered for calculating the average $Q$. The redundancy level can be increased to cater for the increasing fault rates. However, for the isolated chambers as shown in Table 4, the $Q$ value falls below 35% when the fault rates go up to $(40, 20, 20)$, even with a placement redundancy level of 8. For the virtual chambers, the results are shown in Table 5. At the placement redundancy level 8, $Q$ goes as low as 35% at the fault rates $(30, 15, 15)$.

The results show that the mixed redundancy scheme (both placement and control) provides the best results.

### VI. CONCLUSION

In this paper we have presented a modeling and simulation framework for the cell culture microfluidic biochip ProCell. We have proposed a biochip architecture model and a comprehensive fault model capturing permanent faults that occur during the chip operation. Using the proposed simulation framework, we have carried out an architectural level evaluation of two cell culture chamber implementations. The proposed approach considers the fault model, cell placement scheme, fault occurrence rates, and the redundancy level and provides a qualitative success metric as a measure of performance for both architectures in the presence of partial failures. Three different cell placement schemes have been used to evaluate the architectural performance under different redundancy levels and varying fault rates. Our results show that significant improvements in efficiency can be obtained by using both placement and control schemes; increasing the system productivity, saving time and reducing costs. However, evaluation depicts that both architectures have separate limits of maximum tolerable fault rates, after which the redundancy is no longer effective in improving the chip performance.
REFERENCES


Appendices

Appendix 7

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A USER-FRIENDLY, SELF-CONTAINED, PROGRAMMABLE MICROFLUIDIC CELL CULTURE SYSTEM FOR HIGH QUALITY MICROSCOPY

Peder Skafte-Pedersen1, David Sabourin1, Mette Hemmingsen1, Peter Friis Østergaard1, Felician Stefan Blaga2, and Martin Dufva1*

1Department of Micro- and Nanotechnology, Technical University of Denmark, DENMARK and
2 Department of Informatics and Mathematical Modelling, Technical University of Denmark, DENMARK

ABSTRACT

We present a fully self-contained system for long-term cell culture with real-time, high quality microscopic access and programming capabilities for constant perfusion live-cell assays. When assembled, the system constitutes a closed unit complete with microfluidic culturing chip, reservoirs, interconnections and flow control without the need for external mechanical components. Being designed especially for use in cell labs and to fit into standard motorized stages in inverted life science microscopes the system is user-friendly for operators without a microfluidic background. To demonstrate the system capabilities we show examples of switching between different liquids and long-term cell culture with time-lapse imaging.

KEYWORDS: Long-term cell culture, Microscopy, Microfluidics, Interconnection, Fluid actuation, Cell programming

INTRODUCTION

The use of microfluidic tools in cell culture is often limited by the complexity and need for peripheral components in typical research setups incompatible with standard, sterile cell biology work flows and microscopy [1,2]. This can be particularly pronounced in assays requiring high parallelization or multiplexed operation where external fluid actuation requiring e.g. large amounts of tubing from syringe pumps or pressure controllers can lead to unwanted dead volumes and portability issues thus reducing their suitability for routine work in a laboratory environment. A common way to overcome issues with peripheral components is to integrate fluid actuation directly on the chip [3]. Despite the advantages in reduced total system footprint, response time and swept volume, a drawback of on-chip actuation approach is that systems can become highly specialized thereby limiting their versatility and possibly reducing fabrication yield.

The presented system distinguishes itself from other integrated systems [3,4] by having more fluidic inputs/outputs, accepting polymeric and silicon/glass based chips, not relying of pneumatics for fluidic control and being compatible with unobstructed, high quality real-time transmission and fluorescence microscopy observation. We demonstrate how the system can be used to switch between different liquid inputs, which is necessary for programming purposes, and that cells can be seeded and cultured on-chip in a microscope for several days.

SYSTEM DESIGN

In the present system we have implemented fluid actuation by miniaturized peristaltic pumps of the type presented in [5] modified for direct attachment to a cell culture chip by integrated ball joint interconnection blocks [6] without any intermediate fluidic components. This significantly reduces swept volume and compliance compared to distantly placed volume displacement pumps. Thus, except from the power supply and electrical controller, every component for driving the microfluidic chip is on the system. It can therefore easily be moved from cell culture labs for cell loading to microscopes for observation just by connecting one single electrical cable through a standard DA-15 connector (Fig. 1).

Figure 1: a) The complete platform mounted with chip, eight inlet reservoirs and two outlet reservoirs. Miniaturization of pumps and connections allows these and the observation area to fit between life science objectives and a 26 mm working distance (WD) condenser in an inverted microscope. b) Schematic side view showing fit of central parts between 26 mm WD condenser and 2.9 mm WD objective.

The platform (Fig. 1), consists of three miniaturized 8-channel peristaltic pumps [5], inserts with space for up to 30 reservoirs and a chip holder allowing the exchangeable chip to be readily snapped on to 32 self-aligning fluidic ball joint
interconnections [6] by tightening four stainless steel bolts. The exchangeable microfluidic chips are fabricated by direct micromilling in poly(methyl methacrylate) (PMMA) and contain passive microfluidic networks. Depending on the assay requirements the chips can make use of all or a fraction of the inlets and contain single or multiple fluid channel layers for parallel, serial or combinational operation all using the same standardized interconnection and actuation platform. All chips are designed with a total of 24 inlets in groups of 8 and a group of 8 outlets and have a microscope slide footprint for ease of use and compatibility with standard life science equipment.

The central element of the pumps containing integrated channels and ball joint interconnections is made of injection molded PDMS using a fabrication scheme similar to the ones previously reported in [5] and [6]. This low-cost element can be exchanged easily between experiments to avoid cross contamination.

Pump control is obtained through a single electrical interconnection to a custom-made controller. A VBA script in Zeiss AxioVision 4.8.1 (Carl Zeiss, Germany) has been developed to simultaneously control the pumps and Zeiss Axio Observer microscope (Carl Zeiss, Germany) for automated perfusion, imaging and analysis.

Cell loading is carried out by pipetting cell suspension into integrated wells at the outlet side. A uniform cell seeding is readily achieved upon reversing the flow. Due to the design of wells and channel system no agitation between pipetting steps is required making it easy to load different cell lines in each well. When sterilized and assembled the system is fully enclosed and can be transferred between equipment without risk of contamination.

EXPERIMENTAL

Without on-chip valves the switching between compounds must be accomplished by start and stop of the low-compliance pumps, which entails a diffusion limited leakage from channel intersections to the downstream culturing chambers. This, however, can be controlled by design to be without physiological significance.

To test the switching capabilities chips containing T-junctions have been fabricated with inlets connected to two pumps. One inlet feeds a fluorescent diluted Streptavidin-Cy3 (S-Cy3) solution (S6402, Sigma-Aldrich) and the other a nonfluorescent phosphate buffered saline (PBS) solution (P5368, Sigma-Aldrich). The switch rate is determined by measuring the average pixel intensity of the fluorescent signal downstream the junction after changing pump settings.

Long-term cell culture directly on a microscope is demonstrated using a PMMA chip with culturing chambers having a cross section of 200 µm by 1500 µm. HeLa Tet-On cells (Clontech 631155, Clontech Laboratories, Inc.) have been cultured at an average flow rate of 250 nL/min to test proliferation and viability.

To demonstrate gene expression the line has also been transfected with the fluorescent reporter gene ZsGreen1-DR and cultured under the same flow conditions with added doxycycline at a concentration of 5000 ng/mL. During perfusion the setup is mounted in an inverted microscope (Zeiss Axio Observer) equipped with an incubator to ensure stable temperature and CO2 conditions.

RESULTS

Figure 2 demonstrates how two pumps are used to switch between S-Cy3 and PBS with a downstream diffusion limited leakage. The fluorescent signal is reduced approximately two orders of magnitude within 3 minutes at a flow rate of 250 nL/min in channels with 400x150 µm² cross section.

The result is in fair agreement with time-dependent 2D convection-diffusion finite element (FEM) simulations implemented with a constant inlet velocity in COMSOL Multiphysics 3.4 (COMSOL AB, Sweden). This verifies the system’s switching properties. Switch characteristics can be tailored by changing geometry and flow rate, which has been experimentally verified, and proves the system suitable for cell studies with temporally varied exposure profiles.

Figure 2: Switching sequence between PBS and fluorescent S-Cy3 conjugate in a T-junction with channel cross section of 400x150 µm² at a flow rate of 250 nL/min. Arrows indicate flow settings under a) initial condition and b) 8 s, c) 16 s and d) 112 s after switching. Quantification by background corrected, relative pixel intensity over three repetitions and theoretical data based on a FEM simulations is given in e). The box annotation in a) indicates the analysis region.

Figure 3 shows a long-term time-lapse series of HeLa Tet-On cells cultured an average flow rate of 250 nL/min per chamber. The proliferation and adhesion proves the seeding and perfusion method suitable for the application.
A gene expression assay is given in Fig 4, which shows a fluorescent time lapse series of transfected HeLa Tet-On cells stimulated with doxycycline over 36 hours initiated after 5 hours of low flow without doxycycline. The signal is seen to increase during the course of the experiment thus demonstrating a well functioning gene expression.

CONCLUSION

We have presented a complete, self-contained platform for programmable long-term cell culture with microscope access. Using the platform we have demonstrated that two different fluids can be exchanged with two orders of magnitude within few minutes at relevant culturing flow rates and that cells can be cultured and stimulated over days with the system mounted in a microscope.

ACKNOWLEDGEMENTS

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Conference proceeding 4

FAST AND SIMPLE: RECONFIGURABLE ELEMENTS AND SOLUTIONS FOR CREATING AND DRIVING FLUIDIC NETWORKS

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ABSTRACT
Reconfigurable microfluidic elements including multi-channel interconnection and peristaltic pump components are described. The elements form the basis of a modular microfluidic system which features and provides a small footprint, unobstructed microscopic observation, portability and supports a wide variety of assays. Design, fabrication and characterization of the elements are described, as are both motorized and non-motorized methods to control and actuate fluidic networks supported by the system. Applications using the approach are also presented.

KEYWORDS: Interconnections, Pump, Modular, Reconfigurable

INTRODUCTION
Ease of use, reliability and accessibility remain barriers to the adoption of microfluidic approaches [1, 2]. Practical issues including interconnections, pump capacity and control, and suitability to microscopic observation often complicate microfluidic setups. We present modular interconnection and pump components which address these issues and provide simple, reliable and multiple parallel chip-to-world and chip-to-chip interconnectivity and pump capacity. Additionally, for non-experts developing microfluidic investigations there is also a lack of accessible and easily implementable control and actuation solutions. To circumvent this issue, a hand crank is used for simple applications requiring simple fluidic actuation or the commercial LEGO® Mindstorms™ kit is used to program and control pumps when higher degrees of automation are required.

DESIGN AND FABRICATION
Microfluidic ribbons (µFRs) (Figure 1) provide interconnectivity between system components. Each µFR is cast from PDMS and contains eight integrated channels with 240 µm inner diameters spaced 2.25 mm centre-to-centre (1536 well plate standard). Three µFR versions eliminate further tubing requirements. Type I µFRs permit direct chip-to-chip or chip-to-reservoir connectivity. Type II µFRs connect microfluidic components through a further miniaturized and improved version of an 8-channel micro-peristaltic pump (see below, Figure 2) we previously described [3]. Type III µFRs provide chip-to-waste flow (not shown). Faces and integrated channels of µFRs which link components together contain ball joint features (Figure 1) which provide self-aligning, minimal dead volume sealed interconnections [4]. Screws are used to connect elements together (Figure 2) and provide the compressive force which yields interconnection supporting pressures greater than 200 kPa [4], the threshold applicable to most microfluidic applications [5]. All µFRs are manufactured from micromilled PMMA moulds in a manner previously described [4].

Figure 1. Modular microfluidic system components. i) TYPE I µFR containing 8 integrated 240 µm inner diameter channels. Channels filled with dye to facilitate visualization. All channels are bookended by Ball Joint interconnection features. ii) Ball Joint interconnection feature. iii) Type II µFR used for connecting microfluidic chips through a miniaturized peristaltic pump. Channel dimensions and interconnection features are identical to Type I µFR. iv) The peristaltic pump’s multi-roller (MR) is made from eight free-rolling 2 mm stainless steel rods. v) A Type II µFR is placed across the pump’s multi-roller to deliver fluids. A covering piece compresses the Type 2 MR against the multi-roller to pump fluids (not shown).

Type II µFRs are used in a peristaltic micropump (Figure 1 and 2). Type II µFR integrated channels run within raised tubing-like features. When the pump is assembled these tubings are occluded between the micropump’s multi-roller (MR) and a rigid polymeric piece complementary to the MR, thus permitting peristaltic flow in eight individual channels. Pump dimensions, excluding motor, measure only 20 mm (h) x 20 mm (w) x 40 mm (l). For simple microfluidic analyses the micropump can be operated with a hand crank (Figure 3). For more involved assays, motors for and control of micropumps can be provided by the LEGO® Mindstorms™ kit (Figure 3).

EXPERIMENTAL
As interconnection performance was previously assessed [4], the primary concern was pump characterization. To assess Type II µFR durability and micropump performance a microfluidic chip, made from micromilled PMMA, was

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interfaced to two micropumps (Figure 3). The microfluidic chip permitted per rotation displaced volume measurements in each of the eight micropump channels. Mindstorms™ motors were programmed to complete 20 rotations and per rotation displaced volumes were determined by analyzing ‘before’ and ‘after’ photographs. Between measurements motors were programmed to complete 10 000 rotations.

RESULTS AND DISCUSSION

System components are reconfigurable and permit many possible configurations (Figure 2) and higher interconnection numbers than previous reports [4], i.e. up to 32 interconnections on a 35 mm x 35 mm chip, are possible. The Type I and II μFR’s reduce dead volumes between microfluidic chips and within the pump to approximately 1 and 2.25 μL respectively.

Micropump durability testing indicated that all eight channels of Type II uFRs remained functional following more than 63 000 rotations. For all test points, per rotation displaced volumes averaged near 0.7 uL per channel. Average micropump displaced volumes were stable throughout durability testing and differed by approximately +/- 5%. The average coefficient of variance (%CV) of per rotation displaced volume across the 8 channels of a single micropump was 6%, with observed minimum and maximum values of 4.2 and 8.7%. At 63 000 rotations, durability testing was deliberately stopped. Inspection of the Type II uFRs did not reveal any damage. The micropump can thus support extended experiments, such as perfused cell culture, for periods of greater than 3 weeks at flows near 1 μL/min. Comparisons to similar miniaturized peristaltic pumps are not possible as these do not provide such data or do not provide multiple channels [6, 7].

For simple experiments or use in resource poor and remote locations, hand cranks are attached to the micropumps to deliver fluids. A hand crank system (Figure 3) was used to genotype patients for mutations in the β-globin gene (Figure 4).
within a disposable microfluidic microarray-containing PMMA device [8]. For more complicated or longer term assays, the LEGO® Mindstorms™ kit components can be used. The low-cost product (approximately 350 USD) includes motors, a controller, power source and a “child friendly” graphical computer interface. The LEGO® motors provide large flow rate ranges. Using these motors, we have realized flows between approximately 0.033 and 60 µL min⁻¹. As motor rotation can be controlled to 1°, multiple pumps can be accurately activated to time, meter and flow fluids through chips. Cell culture of HeLa cells within a PMMA chip and associated processing steps including, sterilization and loading, were performed using a system actuated by the LEGO® components (Figure 4).

Figure 4. i) Image of fluorescently-detected allele-specific hybridization (ASH) assay used to genotype a patient for mutations in the β-globin gene. A PMMA microfluidic chip containing ASH microarrays and the hand crank system shown in Figure 3(i) were used to process patient material. The patient shown was wild-type (WT) for all interrogated sites with the exception of site 4, for which they were heterozygous mutant type (MT), and therefore carry a mutation associated with a β–globin deficiency. Spot sizes are approximately 250 µm. ii) Image of HeLa cells cultured using a PMMA chip and a system configuration similar to that shown in Figure 2(iii). Fluidic control and assorted operations including sterilization and cell seeding were achieved by LEGO® Mindstorms™ motors and control.

We believe our approach to be more accessible than similar solutions, including Braille displays [6]. The system’s modular components and compact design allows construction of setups that fit on microscope stages or similar life science instruments, thus making it suitable for experiments involving kinetic studies of molecular interactions (e.g., DNA hybridization) and cells (e.g., growth, migration, gene expression). As multiple pumps can be connected to the same device without extensively increasing system footprint, highly multiplexed assays are possible.

CONCLUSION
A portable, modular microfluidic system including methods to actuate and control multiple flow lines has been designed and characterized. The system’s small footprint, modularity, integration, planar interconnections and simple operation make it well suited to a variety of microfluidic applications such as cell culturing and biochemical assays.

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