Microfluidic platforms to access molecular mechanotransduction at the cellular level \textit{in vitro} and \textit{in vivo}

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

FELIX KURTH
Dipl.-Ing. Bioengineering, Technical University of Dortmund

born on 19.01.1982

citizen of the Federal Republic of Germany

accepted on the recommendation of

Prof. Dr. Petra S. Dittrich
Prof. Dr. Ralph Müller
Dr. Alfredo Franco-Obregón

2015
Dissertation

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Felix Kurth
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Abstract

All cells in nature respond to mechanical stimulation. They do not react non-specifically, however. In fact, cell developmental programs are exactly designed to the perceived mechanical forces. That is, rather simple mechanical stimuli induce appropriate but complex responses that result in (apparent) changes in tissue adaptation. These developmental programs are manifold and entail muscle growth under physical stress, bone formation under mechanical load, or the guidance of complex cell fate decisions as in the differentiation of stem cells among others. The link between the impinging external forces and the cells’ mechano-responses is mechanotransduction, which translates the physical into a biochemical signal. In this work, microfluidic platforms were developed to monitor these mechanotransduction processes at the cellular and molecular level.

Many of these transduction events from the physical stimulus into a biochemical signal are mediated by mechanosensitive cation channels of the Transient Receptor Potential (TRP) class, which particularly induce changes in intracellular calcium levels that serve as a second messenger (biochemical signal) and stipulate multiple reaction cascades on demand. Myogenesis, i.e. the formation of muscle tissue, inherently relies on calcium signaling, both during early proliferation of the muscle stem cell pool as well as during contraction of adult muscle fibers. Anomalies in calcium signaling and consequently in TRP channel function have been associated with pathophysiological states and have therefore emerged as an important research field and pharmacological target. Still, current state-of-the-art technologies for assessing TRP channels lack throughput and have, to a certain extent, only limited significance for whole cell responses on a population level. The presented study accordingly envisaged a platform resolving these limitations. Microfluidic technology has proven suitable for cell analyses at the single cell level and provides the opportunity for higher throughput studies. A microfluidic chip device was thus developed that in conjunction with a specifically engineered stage perfusion incubation chamber allowed for the culture and controlled mechanical stimulation of murine myoblasts (C2C12). Highly precise mechanical stimulation was achieved with flow-induced shear forces, and cell mechano-response readout was successfully accomplished with fluorescence microscopy in conjunction with a calcium-sensitive fluorophore. The established platform enabled the evaluation of mechanically-mediated calcium entry under an unprecedented variability of environmental conditions, while still persisting the capability to reliably ascribe channel gating activity to specific TRP channels. By targeted pharmacological modulation of TRP channel activity and parallel determination of channel expression levels the TRP vanilloid 2 (TRPV2) channel was ascribed a role in mediating calcium entry in C2C12 cells under fluid shear stress. The recruitment of TRPV2 was specifically primed by environmental factors, namely low media serum concentrations as well as laminin residing as part of the extracellular matrix. The developed approach thus even permitted inferences on mechanistic TRPV2 gating, which suggests to be regulated by association with the dystrophin glycoprotein complex. The developed microfluidic platform met the initially set criteria facilitating the whole-cell activation and monitoring of mechanosensitive TRP channels within small communities of cells. Moreover, the derived experimental methodology evolved into an expansion to current technologies, such
as the patch clamp, as it allowed for the identification of new roles for particular TRP channels under complex environmental conditions, which might have not been unraveled otherwise.

Bone adaptation is another tissue development that inherently depends on mechanical input. Upon dynamic mechanical loading bone is formed in areas in need of higher stability as well as resorbed from areas of low mechanical strain. Residing within the bone matrix, osteocytes orchestrate osteoclasts to resorb bone as well as osteoblasts to form bone, all in direct correlation to the sensed strain energy. Although much about the regulatory pathways in bone remodeling is known, investigations including these highly localized transduction processes only recently allowed for a first quantitative description of bone adaptation. Still, current bench-top approaches retrieve only rare data sets from cell samples dissected out of these tissue sections. As microfluidic technology has the potential to augment this data density derived from these precious samples, an experimental strategy was developed that permitted the transfer of dissected cell samples from bone cryosections into a microfluidic system. Cell dissection and transfer was accomplished by laser capture microdissection. The strategy was implemented by the use of a specific chip design allowing to be closed after sample capture and operated identical to a conventional microfluidic device. After chip assembly, the microfluidic platform was utilized for sample preparation steps and arranged for the analysis of gene expression patterns by reverse transcription and quantitative polymerase chain reaction (RT-qPCR). First RT-qPCR protocols were established and tested in conjunction with fluorescence microscopy and specially designed temperature control.

In summary, this work presents the development, characterization, and successful application of microfluidic platforms for the investigation of cell mechanotransduction processes at the cellular and molecular level. The results achieved herein will assist in shaping a better understanding of myogenesis to counteract pathophysiological muscle conditions due to aging, trauma, disuse or disease. Moreover, the demonstrated versatility of microfluidic technology will encourage future studies to tackle specific mechanobiological objectives as a result of the options microfluidic technology is able to offer.
Zusammenfassung


Acknowledgements

This work would have never reached its final state without the support and help of many people. First, I would like to thank Prof. Dr. Petra S. Dittrich for the opportunity to work on my PhD thesis in her research group. I especially appreciate her trust in my work during the first year, in which my PhD status at ETH was still pending for acceptance, and during the final stage of my PhD, in which the manuscript on TRP channels took shape. Second, I thank Prof. Dr. Ralph Müller for agreeing to co-supervise my PhD thesis and his support throughout the collaboration project. I gratefully acknowledge support, scientific input, and motivation from Dr. Alfredo Franco-Obregón, without whom the scientific merit in cell physiology in chapter 5 would have not been achieved. I very much appreciate short-termed skype meetings with him, helpful discussions, and support beyond the “conventional” measure. Further, I thank all three for scientific guidance and very helpful corrections during writing of manuscripts and the thesis.

Next, I would like to thank all additional scientific co-authors of the manuscripts: (i) Robin E. Wilson, Andreas J. Trüssel and Dr. Duncan J. Webster for fruitful discussions, support in experimental procedures and tremendous work motivation; (ii) Christoph A. Bärtschi for the development and fabrication of the stage perfusion incubation chamber; (iii) Simon K. Küster, Marco Casarosa and Dr. Karin Wuetz-Kozak for technical support, scientific input and experimental work; (iv) Dr. Klaus Eyer for biological expertise and exploring his artistic skills.

In the context of travel grants and support funding, I highly appreciate funding from the Swiss Chemical Society and from the Gesellschaft Deutscher Chemiker.

Very special thanks go to all (former) members of the Dittrich lab, Dr. Daniel F. Schaffhauser, Dr. Benjamin Z. Cvetković, Dr. Josep Puigmarti-Luis, Dr. Phillip Kuhn, Dr. Klaus Eyer, Dr. Tom Robinson, Dr. Simon K. Küster, Dr. Andriu Cavegn, Bernhard Sebastian, Pascal E. Verboket, Simone A. Stratz, Mario Lenz, Dr. Nikolaus Naredi-Rainer, Dr. Dario Lombardi, Dr. Andreas Jahn, Dr. Eva Bönzli, Dr. Maik Hadorn, Dr. Conni Hanke, Yannick Schmid, Dominik Hümmer, Simon Bachler, Lucas Armbrrecht and all the Semester/Bachelor/Master students. Thank you very much for the gorgeous time at work and anywhere else, for inspiring discussions, technical and scientific support, and coffee breaks. I highly appreciate the opportunity to collaborate with Andreas J. Trüssel, his help, outstanding enthusiasm, and of course the best beer from Switzerland. In line with the collaboration with the Biomechanics Group I would specially like to thank Robin E. Wilson for her exceptional motivation to achieve progress in this intense project. Further, I gratefully acknowledge all work conducted by the mechanical workshop, in particular by Christoph A. Bärtschi, but also by Christian Marro.

Special thanks go to those who additionally proofread this thesis, namely Dr. Benjamin Z. Cvetković, Andreas J. Trüssel, Dr. Florian Kurth, and Dr. Daniel F. Schaffhauser.

I very much appreciate support, scientific guidance, and educational input during the lab course “Biological Chemistry A” by Prof. Dr. Peter Kast, Dr. Kathrin Roderer, and Dr. Richard Obexer among others. I would like to take the chance to thank all people working at the D-CHAB institute (known and unknown, professors, technicians, students and post-docs), who take care to provide an extraordinary work environment.
Many thanks go to all my friends who supported me either in the scientific context or by dragging me out of the lab and stood by my side when times got rough, sad or when occasions had to be celebrated. So thank you Daniel, Ben, Kim, Josep, Juani, Bernie, Tom, Klaus, Phil (the award is still alive!), Andriu, Robert, Rob, Steffi, Andreas, Toni, Robertas, Bruno, Hendrik, André, Anne, Ernie, Jörg, Marcel, and all others I do not mention – you know who you are!

This work would have not reached its final state without the endless support by Ina Sommer. Thank you so much for all that you gave and all that you are.

Last but not least, I particularly like to thank my family, who enabled me to take this opportunity. My parents further for the numerous visits to Zurich (we will leave “tomorrow”) and my brother for skiing the Sierra, snorkeling the reef, and epic green days in the Alps.
### Nomenclature

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(c)DNA</td>
<td>(complementary) Deoxyribonucleic acid</td>
</tr>
<tr>
<td>(h)MSC</td>
<td>(human) Mesenchymal stem cell</td>
</tr>
<tr>
<td>(m)RNA</td>
<td>(messenger) Ribonucleic acid</td>
</tr>
<tr>
<td>(n)DEP</td>
<td>(negative) dielectrophoresis</td>
</tr>
<tr>
<td>2-APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>AEAPS</td>
<td>Aminoethylaminopropyltrimethoxysilane</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>DGC</td>
<td>Dystrophin glycoprotein complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ETFE</td>
<td>Ethylene-tetrafluoroethylene</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FEP</td>
<td>Fluorinated ethylene propylene</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fluo-4 AM</td>
<td>Fluo-4 acetoxyethyl methyl ester</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence (Förster) resonance energy transfer</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisiloxane</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HPTS</td>
<td>8-hydroxypyrene-1,3,6-trisulfonic acid</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGF-1Ea</td>
<td>Insulin-like growth factor 1Ea (splicing variant of IGF-1)</td>
</tr>
<tr>
<td>IGF-1Eb</td>
<td>Insulin-like growth factor 1Eb (splicing variant of IGF-1)</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LN</td>
<td>Laminin</td>
</tr>
<tr>
<td>mdx</td>
<td>Skeletal muscle lacking dystrophin</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte enhancer factor-2</td>
</tr>
<tr>
<td>MEMO</td>
<td>3-methacryloxypropyltrimethoxysilane</td>
</tr>
<tr>
<td>MGF</td>
<td>Mechano growth factor (splicing variant of IGF-1)</td>
</tr>
<tr>
<td>MSD</td>
<td>Mean squared displacement</td>
</tr>
<tr>
<td>ND</td>
<td>Neutral density</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly-(dimethylsiloxane)</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyether ether ketone</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLL-g-PEG</td>
<td>Poly(L-lysine)-grafted poly(ethylene glycol)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>POM</td>
<td>Polyoxymethylene</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RR</td>
<td>Ruthenium red</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription followed by quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SKF-96365</td>
<td>1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole</td>
</tr>
<tr>
<td>SOC</td>
<td>Store-operated channel</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPA</td>
<td>Transient receptor potential ankyrin</td>
</tr>
<tr>
<td>TRPC</td>
<td>Transient receptor potential canonical</td>
</tr>
<tr>
<td>TRPM</td>
<td>Transient receptor potential melastatin</td>
</tr>
<tr>
<td>TRPML</td>
<td>Transient receptor potential mucolipin</td>
</tr>
<tr>
<td>TRPN</td>
<td>Transient receptor potential NOMPC</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>------------------------------------</td>
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<tr>
<td>TRPP</td>
<td>Transient receptor potential polycystin</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>µTAS</td>
<td>Micro total analysis system</td>
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### List of prefixes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Prefix</th>
<th>Exponential Notation</th>
<th>Factor</th>
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<tbody>
<tr>
<td>G</td>
<td>giga</td>
<td>$10^3$</td>
<td>$10^9$</td>
</tr>
<tr>
<td>M</td>
<td>mega</td>
<td>$10^3$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>c</td>
<td>centi</td>
<td>$10^2$</td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
<td>$10^{-1}$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
<td>$10^{-2}$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
<td>$10^{-3}$</td>
<td>$10^9$</td>
</tr>
<tr>
<td>p</td>
<td>pico</td>
<td>$10^{-4}$</td>
<td>$10^{12}$</td>
</tr>
<tr>
<td>f</td>
<td>femto</td>
<td>$10^{-5}$</td>
<td>$10^{15}$</td>
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### List of volumes in liter

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<tbody>
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<td>l</td>
<td>liter</td>
<td>$1 \text{ dm}^3$</td>
<td>$10^{-3}$ l</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
<td>$1 \text{ cm}^3$</td>
<td>$10^{-3}$ l</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
<td>$1 \text{ mm}^3$</td>
<td>$10^{-6}$ l</td>
</tr>
<tr>
<td>nl</td>
<td>nanoliter</td>
<td>$(100 \mu\text{m})^3$</td>
<td>$10^{-9}$ l</td>
</tr>
<tr>
<td>pl</td>
<td>picoliter</td>
<td>$(10 \mu\text{m})^3$</td>
<td>$10^{-12}$ l</td>
</tr>
<tr>
<td>fl</td>
<td>femtoliter</td>
<td>$1 \mu\text{m}^3$</td>
<td>$10^{-15}$ l</td>
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### List of non-SI derived units

<table>
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<tr>
<td>dyn</td>
<td>dyne</td>
<td>$1 \cdot 10^{-5}$ N</td>
<td>$1 \cdot 10^{-5}$ kg·m/s²</td>
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</tbody>
</table>
Chapter 1

**General introduction**
1.1 Mechanobiology

Most cells respond to mechanical stimulation (1). Cellular mechanosensitivity has for a long time been appraised as an epiphenomenon of cell culturing. In fact, in recent years cellular mechanosensitivity has been identified as a fundamental determinant factor in tissue development, which in addition is dynamically-regulated to alter developmental outcome (2, 3). Mechanical forces intrinsically help govern cell fate decisions, e.g. on stem cell lineage and are also involved in the developments of diseases (4-6).

Mechanical stimulations are induced by a vast variety of sources. They occur as compression, stretch and shear forces, and gravity. Gravity displays the most prominent of them as it impinges permanently onto all cells on the planet. Altering gravity elicits transient changes, such as the very specific transport activity of mechanosensitive cation channels (7), as well as modifies tissue developmental programs leading to muscle loss under microgravity (8, 9). These effects certainly only appear under extraordinary circumstances, for instance during space travel. However, they underline the fundamental paradigm of mechanobiology, i.e. particular forces imparting on cells are preconditioned for a relevant cell fate. Apart from space travel, muscle and bone, two of our most prominent body tissues, adapt to mechanical forces (10, 11). For instance, muscle growth ceases in periods of minimal physical activity (12) and bone mass forms under repeated dynamic physical loading (13, 14).

The process whereby mechanobiological processes act at the cellular level is known as mechanotransduction. Mechanotransduction is defined as how physical forces are translated into biochemical signals. These translation processes are on the one hand species dependent as well as underlie a plethora of molecular options for mechanotransduction, such as membrane transducers or conformational changes in the cell cytoskeleton or integrin binding complexes among others (15, 16). All mechanobiological events are characterized by the same cycle, namely initial mechanosensing followed by mechanotransduction and a final mechanoresponse. Figure 1.1 illustrates the hierarchical structure and dimensional relations in mechanobiology commencing at the macro-size and continuing down to the molecular level at which the physical forces are translated into biochemical signals that, in turn, result in transient or permanent adaptations at the nano-scale as well as at larger dimensions. The following sections discuss the process of myogenesis (chapter 1.1.1). Bone mechanobiology is discussed in chapter 6. As mechanotransduction events depict a quantity of signal transduction events and developmental outcome too large to be summarized briefly, excellent reviews and manuscripts exist that provide cell species dependent descriptions of mechanobiological developments, for instance on general mechanobiology (17-21) and their effects on tendon (22), cartilage (23, 24), angiogenesis (25-27) and connective tissue (28).

1.1.1 Myogenesis

Within the time course of muscle formation, i.e. myogenesis, muscle cells undergo multiple changes in phenotypes unlike all other cell types. Muscle tissue comprised of numerous muscle fibers is a result of a complex formation process starting with single-nucleated muscle progenitor cells that pass through a series of proliferation, differentiation and maturation that ultimately
generates functional muscle present in our body (29, 30). Three different specialized forms of muscle exist, namely cardiac, smooth and skeletal muscle. Cardiac myocytes build heart muscle and elicit the electrical pulses via which the heart rate is controlled. Smooth muscle cells are predominantly found as an essential part of blood vessels generating the necessary transient vessel constrictions and openings. Skeletal muscle tissue is our most abundant tissue and is also referred to as striated muscle or voluntarily controlled muscle. The following description of myogenesis refers to the development of skeletal muscle.

**Fig. 1.1:** Mechanical factors impinge on progressively smaller scales down to the molecular level where they are translated into biochemical signals (mechanotransduction). The signal transduction is manifold and in many cases subdued to changes in protein conformation, which encompass not only but to a large extend membrane transducers, such as receptor or adhesion proteins and mechanosensitive cation channels among others. The emerging molecular signaling, e.g. changes in cytosolic calcium levels, cascades succeeding reactions that result in distinctive gene expression levels as well as intracellular signaling without transcriptional changes. From the intersection of these highly convoluted signaling pathways directed signaling entails the intracellular conversion that traverses the length scales upward, whereby the distinct changes may affect the cell itself (autocrine signaling) as well as cause developmental programs at the larger scale (endocrine signaling). Adapted from (11).
**Fig. 1.2:** Principal time course of *in vitro* myogenesis for skeletal muscle formation and regeneration. (A) Precursor muscle cells originate from mesenchymal stem cells by directed differentiation. (B) Further cell determination by myogenic regulatory factors yields the myoblasts, the muscle stem cells. Myoblasts are stimulated to proliferate by means of mechanical stimulation and growth factors. (C) The first step in muscle formation in the embryo is the alignment and fusion of myoblasts forming multinucleated and terminally differentiated myofibers. (D) The alignment of multiple myofibers results in the formation of mature primary myofibrils. For further growth or regeneration postnatally, satellite muscle stem cells undergo proliferation and final fusion with the myofibers and/or themselves thereby building new myofibers.

Myoblasts are derived from mesenchymal stem cells by directed differentiation via somitogenesis under the control of various differentiation (e.g. Wnt) and transcription factors (e.g. MyoD and myf5) (30). To form the primary myofibers, the muscle stem cells (i.e. the myoblasts) align and fuse to each other resulting in terminally differentiated, multi-nucleated myofibers. Particular alignment of these myofibers assembles the myofibrils, which finally are the basis of mature muscle tissue. The whole process of myogenesis is illustrated in figure 1.2.

Adult myofibers are terminally differentiated and thus lack the capability to expand (muscle growth) as well as regenerate from trauma. In 1961, muscle satellite cells located between the sarcolemma and the basal lamina of adult myofibers were identified (31), which turned out to accomplish these two fundamental tasks. In the unperturbed state the satellite cells remain quiescent, i.e. they do not proliferate. Mechanical stimulation, such as muscle activity or myotrauma, activates them to initiate proliferation, migration to the damaged regions, and fusing to existing myofibers (hypertrophy) or possibly fusing to build new myofibers (hyperplasia). Proliferation is regulated by different growth factors, i.e. growth factor beta, insulin-like growth factor 1 (IGF-1) and the fibroblast growth factor (32) as well as specialized integrin binding sites (33). Some of the satellite cells undergo a process of self-renewal by reestablishing the quiescent state and thereby providing the necessary satellite cell pool for future regeneration cycles (30).

**Myoblast proliferation is calcium dependent**

Myoblast proliferation and fusion are two reoccurring processes in myogenesis, which constitute growth and formation of muscle and endow myogenesis with its unique physiological attributes. As the focus in this thesis is laid on the time course and mechanistic regulations during myoblast proliferation, the following section briefly discusses relevant molecular conditions controlling this early stage of myogenesis.
Myoblast proliferation is a calcium dependent process (10, 35). Myogenic satellite cells sense mechanical stimulation via mechanosensitive membrane incorporated cation channels of the Transient Receptor Potential (TRP) class (cf. chapter 1.1.2). These TRP channels support calcium entry into the cytosol (36, 37) as well as induce calcium release from intracellular stores (calcium induced calcium release; CICR) (38). Further increases in the cytosolic calcium concentration are induced by surface membrane associated store-operated channels (SOC), which are activated by depletion of the sarcoplasmic calcium stores (39) (Fig. 1.3.A). There are some indications that TRP channels act as mechanosensitive channels as well as SOCs (38-42). The elevated calcium levels activate a signaling cascade via calcineurin leading to a dephosphorylation of the nuclear factor of activated T cells (NFAT) that can afterwards enter into the nucleus (Fig. 1.3.A) (10). The translocation subsequently results in transcription and subsequent alternative splicing of the muscle remodeling genes, particularly the insulin growth factor 1 (IGF-1; Fig. 1.3B) gene. More specifically, enhanced mechanical stimulation yields the mechano growth factor (MGF) inducing myoblast proliferation, whereas resting mechanical stress accounts for the systemic IFG-1 isoforms IGF-1Ea and IGF-1Eb, both guiding myoblasts into terminal differentiation (43). Further signaling cascades leading to the dynamic regulation of the muscle remodeling genes are for example the calcium dependent myocyte enhancer factor-2 (MEF2) transcription factor as well as the activation of AMP-activated protein kinase (AMPK) upon ATP depletion due to muscle workload (10).
Fig. 1.4: The architecture of TRP channels. TRP channels comprise of 6 transmembrane domains (S1 – S6). The pore for cations to pass the membrane is located between S5 and S6. This transport can be blocked by antagonists, such as lanthanum ions (La\(^{3+}\)) or ruthenium red (RR), as well as fortified or elicited by agonists, e.g. 2-aminoethoxydiphenyl borate (2-APB). The N-terminal (N) and C-terminal (C) ends are both oriented into the cytosol and constitute various binding elements. The dystrophin glycoprotein (DG) complex in particular binds to the C-terminal end of selective TRP channels to mediate mechanical forces through the actin cytoskeleton and extracellular matrix proteins. Further, sarcolemma components contribute to channel gating activity, as is the case for phosphatidylinositol-4,5-bisphosphate (PiP\(_2\)). Adapted by permission from Macmillan Publishers Ltd: (44), copyright (2001).

1.1.2 Transient Receptor Potential channels

It is no surprise that cells respond to changes in their environment. The way they sense these changes and commence transient or permanent adaptation is versatile. A unique role in the signal transduction process upon impinging stimuli is mediated by cation channels of the Transient Receptor Potential (TRP) class. The diversity in activity and mechanisms of activation of the TRP channel superfamily is unmatched by any other group of trans-membrane ion channels. TRP channels initiate signaling cascades critical in sensory physiology with contributions to mechano-, thermo- and osmosensation as well as are of fundamental relevance to vision, taste, touch, hearing and olfaction (45). Equipped with these sensory capabilities cells master sensing and directed responding to (dynamic) changes in their local environment, which for many cells is of vital significance in their developmental programs. TRP channels are further involved in calcium and magnesium homeostasis, where they incur the assignment of active cation gating. Besides sensing the “outside world” TRP channels have adapted to respond to stimuli from within the cell as well such as in response to calcium release via the ryanodine receptors or inositol 3-phosphate receptors of the endoplasmic (sarcoplasmic) reticulum (46) as well as by actin tension due to cell migration (47).

Though a plethora of polymodal functional roles has been ascribed to TRP channels in normal cell development, incorrect channel gating contributes to pathophysiological mechanisms (48). These latter contributions have evoked increasing pharmacological interest within the last 15 years as the occurring diseases concerned by channel dysfunctions (channelopathies) span a wide range commencing with intestinal, respiratory and cardiovascular diseases and continue to neurodegenerative and muscle disorders, just to name a few.
Tab. 1.1: The TPR channel superfamily. Each subfamily consists of different homologs; some of them are species dependent. Numbers taken from (49). Reprinted with permission from AAAS.

<table>
<thead>
<tr>
<th>TRP subfamily</th>
<th>Homologs</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canonical</td>
<td>7</td>
<td>human (6), mice (7), flies (3), worms (3)</td>
</tr>
<tr>
<td>Vanilloid</td>
<td>6</td>
<td>human (6), mice (6), flies (2), worms (5)</td>
</tr>
<tr>
<td>Melastatin</td>
<td>8</td>
<td>human (8), mice (8), flies (1), worms (4)</td>
</tr>
<tr>
<td>Ankyrin</td>
<td>4</td>
<td>human (1), mice (1), flies (4), worms (2)</td>
</tr>
<tr>
<td>NOMPC</td>
<td>1</td>
<td>Not found in mammals</td>
</tr>
<tr>
<td>Polycystin</td>
<td>3</td>
<td>human (3), mice (3), flies (1), worms (1)</td>
</tr>
<tr>
<td>Mucolipin</td>
<td>3</td>
<td>human (3), mice (3), flies (1), worms (1)</td>
</tr>
<tr>
<td>Yeast*</td>
<td>1</td>
<td>yeast only</td>
</tr>
</tbody>
</table>

* Distantly related, normally not listed.

TRP channels are thought to be comprised of six transmembrane domains that assemble into tetramers thereby forming non-selective cation channels (46, 50). The channel pore is located between the transmembrane subunit 5 (S5) and S6. The N and C-terminal termini are attached to the S1 and S6 domains, respectively (Fig. 1.4) (44, 50). Both are located in the cytosol and contain functional units that can be affected by molecular complexes modulating channel gating. For instance, the C-terminal end has been shown to associate with the dystrophin glycoprotein complex through which membrane and cytoskeletal stretch facilitate channel opening (51). Another mechanism controlling TRP channel activation depends on the lipid composition in the inner leaflet of the sarcolemma. The rare phospholipid phosphatidylinositol-4,5-bisphosphate (PiP$_2$) proved to be a necessary cofactor for TRP channels: upon PiP$_2$ depletion, TRP channel activity ceases (52, 53). Although TRP channels are non-selective cation channels transporting calcium, magnesium and sodium, most of them are permeable to calcium. Some of the members of the TRP channel family are to a certain extend cation-selective, e.g. they favor permeation of calcium (44, 54). Channel gating can additionally be manipulated by the administration of channel (specific) agonists and antagonists. The interaction between the agent and the channel may range from transient binding to the channel pore and continue to much more complex interactions (55-58). The principle architecture of TRP channels as well as examples for modulating agents and binding molecular complexes are illustrated in figure 1.4.

In total seven subfamilies of TRP channels exist that have only been fully discovered within the last 15 years (Tab. 1.1). These subfamilies are classified according to their respective amino acid sequence and to a lesser degree functional overlaps (45). The founding member of the TRP superfamily is the Drosophila TRP (59). From this protein sequence, five subfamilies emerged bearing similar sequence homologies and are named according to the initially described member of each subfamily, namely canonical (C), vanilloid (V), melastatin (M), ankyrin (A) and NOMPC (N; no mechanoreceptor potential C). Two more distantly related TRP families exist that in contrary to the first group contain a large loop between the S1 and S2 transmembrane domain (polycystin (P) and mucolipin (ML)). Notably, a distantly related eighth TRP member is
present in yeast (60), however, it is normally not reckoned among the TRP superfamily. Table 1.1 lists all channel subfamilies, each comprising of different amounts of homologs that, in addition, are partially species dependent as well as substantially differ in their physiological relevance.

The particular roles of TRPC1 and TRPV2 in myogenesis are briefly discussed in the following section. More detailed information on the various TRP channel classes is summarized in excellent reviews depicting their functional roles as well as listings of pharmacological modulators for each channel class (44-46, 50, 61-64).

**TRPC1 and TRPV2 in myogenesis**

Both TRPC1 and TRPV2 have been identified with distinct physiologically relevant roles in the time course of myogenesis, with particular contributions of TRPC1 in normal myoblasts and mature myotubes (65) and TRPC1 and TRPV2 in dystrophic myofibers (66-70). This introductory section summarizes their most important known functions in muscle tissue. Further information and discussion on their respective contributions is provided in chapter 5 and in the cited references.

TRPC1 has been the first identified mechanosensitive channel regulating calcium homeostasis in myoblasts and mature skeletal muscle tissue (36, 37). Not only in muscle, but as well in other vertebrate cells TRPC1 proved to constitute the main stretch-activated calcium channel (71). In myogenesis TRPC1 gating is not only activated upon mechanical stretch (36, 37, 51), it moreover initiates calcium release from the sarcoplasmic reticulum (calcium induced calcium release; CICR) (40) and is regulated by store-operated calcium release (store-operated channel; SOC) (39). Physiological relevance of TRPC1 has been ascribed for myoblast proliferation (35, 72), differentiation (73) as well as calcium homeostasis in mature myofibrils (37, 65). The recruitment of TRPC1 dynamically adapts to the grade of mechanical stimulation (35) and developmental stage (72). Furthermore, TRPC1 has been identified to be involved in the pathophysiological mechanism of muscular dystrophy, where the different degrees of TRPC1 levels are suggested to correlate with the different degrees of the pathophysiological phenotype (65, 66).

TRPV2 is the maybe least characterized cation channel of the whole TRP superfamily (74). Though it was assumed for a long time that TRPV2 resembles TRPV1 in its functional repertoire due to their highly similar amino acid sequences and the fact that TRPV2 indeed shares similar sensory tasks regarding nociception, TRPV2 emerged to also sustain unique tasks (74, 75). TRPV2 is an osmosensory (76), PiP2-dependent (77) calcium channel that in myogenesis underlies the regulation by growth factors (78) and mechanical strain (68-70). The mechanical activation of TRPV2 has, however, merely been shown in dystrophic muscle, whereby mechanically-mediated calcium entry via TRPV2 results in abnormally high cytosolic calcium levels, which are considered to contribute to muscle necrosis (79). Particular regard should be given to the fact that TRPV2 displays attributes, which are species dependent and have conspicuous consequences for regulatory mechanisms and pharmacological treatment (80).
1.1.3 State-of-the-art characterization of TRP channels

As discussed beforehand trans-membrane ion channels play important physiological roles in cell development and fate and, since the mid 1990's, have become a highly interesting target for pharmacological studies (57, 81, 82). The assessment of ion channel gating has been first accomplished by the patch clamp technology in the late 1970’s. The evolution of the patch clamp technology has to date attained tremendous achievements, which include the measurement of single-channel currents (83) and the identification of stretch-activated calcium channels (36, 37), which constitute the basis for channel-gated mechanotransduction processes. Combined with molecular biological analyses the activation properties, e.g. voltage gated or mechano-gated, and pharmacology of the known TRP channel classes and sub-types could be identified and explored. Further achievements in the characterization of TRP channels were accomplished by the application of fluorescent dyes, which were either linked to particular targets (84) or used as molecular sensors for transported ions ((85, 86), cf. chapter 1.2). Finally, recent studies combined both methods, i.e. patch clamping and fluorescence microscopy, thereby facilitating for example the monitoring of highly localized channel-mediated calcium transients (87, 88). The following section introduces the principles of the patch clamp technology. The application of fluorescent dyes in mechanobiological studies is summed in chapter 1.2.2 and 1.2.3.

Patch clamp technology

The patch clamp technology is a method to accurately measure trans-membrane ion fluxes attributed to sodium, potassium, calcium and magnesium, resulting from active (and selective) channel gating. It is an electrical measurement method, which relies on the recording of changes in trans-membrane current or changes in trans-membrane potential. To set the imperative in vitro conditions rendering the recording of small currents in the pA-range possible, the to be monitored membrane area is isolated. A glass patch pipette with a very small patch diameter (few to a single micrometer) is brought into contact with the cell membrane. Upon careful suction applied in the patch pipette, the membrane area circumvented by the rim of the pipette is drawn into the barrel of the pipette (Fig. 1.5). The outer rim of the pipette is hereby in close contact with the membrane thereby electrically isolating it from the outer bath solution, also termed the seal. The tightness of the seal dictates the resistance of the seal, which commences at several MΩ for loose suction and continuing up to multiple GΩ for tight membrane bleb enclosure (83).

The resistance of the seal determines the sensitivity of the patch clamp measurement. For example a 100 mV change in the pipette potential drives a “leakage” current of only about 5 pA through a 20 GΩ seal (83). Seals with low resistances require balancing of the pipette potential within less than 1 mV of the bath potential. Otherwise large, noisy leakage currents flow through the seal conductance prohibiting the measurement of single channel currents. Although the GΩ seal allows such a recording, the current generated by the transport of the ion of interest is still very low. To amplify this current monovalent or divalent charge carrier ions, such as barium, are frequently added to the patch solution to fortify the total channel current to a detectable level (89-91). Figure 1.5 illustrates the principle operation of a patch clamp measurement in the voltage clamp actuation mode.
Fig. 1.5: Principle of the cell-attached patch clamp technique. A A patch pipette with a tip diameter of about 1 µm is brought into contact with the cell membrane. These small tip diameters allow for the access of single membrane channels. B Upon suction by the patch pipette the enclosed membrane is drawn into the pipette resembling a membrane bleb that is electrically and chemically isolated from the bath solution. The induced high resistance seal (gigaohm-seal) forms a lateral barrier of ions across the cell membrane. By changing the electrical potential of the patch solution relative to the bath solution (voltage clamp) ion exchange through the membrane channels within the isolated membrane patch occurs, which in turn can be monitored by its current in the pA-range. Current direction is a function of holding voltage applied to the patch and the electrochemical driving for an ion species for which the channel is selective. Adapted from (83) with kind permission from Springer Science and Business Media.

After formation of the seal, different modes of channel actuation exist. Besides the voltage clamp to measure the currents of trafficking ions at a constant holding potential, a defined current can be induced and the resulting change in potential is recorded (i.e. the current clamp). For instance, deviations of the cell’s resting potential as a result of external stimuli are frequently determined by induced zero current and measurements of changes in the membrane potential. The identification of mechanosensitive channels and the quantification of their activity are commonly perceived by the pressure clamp, in which the degree of pipette-induced suction changes the membrane tension in the isolated patch under a constant holding potential. The magnitude of the elicited channel currents hereby coincides with the level of membrane stretch (37, 92). Membrane channel activity can be monitored in isolated membrane patches still attached to a whole cell (whole cell recording) as well as to membrane patches dissected from a cell (outside-out patch or inside-out patch) (83). Cell-free membrane patches permit the investigation of the same membrane patch under the influence of multiple compounds or concentrations, e.g. by changing the bath solution. Not only the bath solution can be altered to induce changing environmental conditions, especially exchanging the pipette solution offers valuable clues to channel gating properties, such as the effect of channel blockers or agonists, and can same time be applied in the whole cell recording paradigm. In contrary to the outer bath solution, the pipette solution cannot be changed during an experiment.

Still, the activity of especially mechanosensitive membrane channels is literally bound to intracellular environmental components. For instance, attachment to the actin cytoskeleton (51, 93) and the inherent dependence on the lipid membrane composition (53) prime channel-mediated mechanotransduction. By the generation of the membrane bleb within the barrel of the
patch pipette both of these fundamental prerequisites for channel integrity are supposed to be disrupted, which evoked controversial discussions within the scientific community on the patch clamp technology in general (94). A way to circumvent this conflict is the use of the whole cell patch clamp (95). In contrary to the patch clamp approach, the outer membrane within the pipette is ruptured by suction, which allows electrical and fluidic access to the entire cytosol. The drawback of this whole cell clamping is that the cell interior has to be dialyzed by necessity. Otherwise the induced current or potential that enable the measurement of the trans-membrane potential and current, respectively, cannot be achieved.

Further variations in mode of actuation and configuration of the patch clamp exist. Details of these particular approaches and their characterization and limitations are well presented in literature (94, 96-99) and not discussed here.
1.2 Fluorescence spectroscopy

The emission of light from any substance that undergoes a change in its electronic state is called luminescence. The return of the substance from an electronically exited state to the ground state occurs by the emission of a photon (100, 101). The time scale on which this electronic state change happens divides luminescence into the two incidences fluorescence and phosphorescence, which are additionally both dependent on the nature of the excited state. To reach an excited state, i.e. an excited singlet state, a molecule in its ground state absorbs energy from an external source, which converts an outer electron into an excited orbital. The return into the ground orbital results in the emission of a photon. Depending on the spin orientation of this electron, the time scale for the conversion back to the ground state changes. In case the spin orientation is paired to the second electron remaining in the ground orbital (opposing spin), the transition occurs rapidly ($10^{-9}$ s) and is termed fluorescence. However, the spin orientation can also be identical for both electrons, and the return to the ground state from the here existing triplet state is forbidden. This leads to emission rates on longer time scales ($>10^{-4}$ s) and is termed phosphorescence. The described light absorption and emission processes are typically illustrated in the Jabłoński diagram (Fig. 1.6A) (86, 102). As depicted in figure 1.6A, the energy of the emission is commonly lower than the energy of absorption. This leads to a shift in wavelength from absorbed to emitted light, i.e. from shorter to longer wavelengths, and is defined as the Stokes’ Shift (Fig. 1.6B) (86, 100). Based on the Stokes’ Shift, the fluorescence detection is very specific as the excitation wavelength can be excluded from the sample signal (cf. chapter 1.2.1).

Besides this high specificity due to precise wavelength selection, fluorescence spectroscopy is superior to other analytical techniques since the fluorescent probe stays intact after the measurement. In contrary to analytical techniques such as mass spectrometry, fluorescence spectroscopy allows for time-lapse monitoring of the same sample. Certainly, fluorescent molecules undergo losses in their efficiency (ratio of absorbed photons to emitted photons) due to e.g. bleaching (100, 103). However, by carful experimental design these effects can either be minimized or the retrieved data can be corrected for the respective effect.

1.2.1 Wavelength selection in fluorescence microscopy

The excitation and emission wavelengths have to be carefully selected for effective fluorescence microscopy. The excitation light is typically chosen close to the excitation maximum to reduce the excitation light energy, which may lead to fast dye bleaching otherwise (86, 101). The excitation light has to be excluded in the fluorescence emission detection, which is commonly achieved by an arrangement of the detector at a 90° or 180° angle relative to the excitation source. This way scattered light, i.e. Rayleigh scattering, can be excluded from the sample signal. Moreover, filter systems are employed that efficiently select the respective wavelengths (Fig. 1.7).
Fluorescence spectroscopy

Fig. 1.6: A Jabłoński diagram: upon photon absorption the fluorescent molecule is brought into the singlet excited state. Due to partial energy dissipation (vibrational relaxation) the emitted fluorescence consists of a longer wavelength than the excitation light. In case of a ‘forbidden’ transition of the outer electron, the molecule reaches the triplet state (intersystem crossing). When a fluorophore returns from the triplet state to the ground state phosphorescence occurs. Adapted by permission from Macmillan Publishers Ltd: (86), copyright 2005. B The spectral characteristics of a fluorophore directly correlate with the energy step size required for a molecule to reach another singlet state. The energy levels for absorption and emission at different wavelengths are shown for the fluorophore fluorescein isothiocyanate (FITC). The Stokes’ Shift is exemplarily indicated for the wavelength change from excitation to emission maxima (lower graph). Adapted by permission from Macmillan Publishers Ltd: (86), copyright 2005.

Fig. 1.7: A. Principle of a filter cube assembly for the selection of excitation and emission light in an inverse microscope: a light source (e.g. mercury lamp) emits a wide spectrum of light (UV to IR). The light can be preselected by a heat filter to remove the IR spectrum or by a neutral density (ND) filter to decrease its intensity (no. 1). The excitation filter selects the fluorescence excitation wavelength (bandpass filter, no. 2), which is reflected onto the sample by a dichroic mirror (no. 3). The emitted fluorescent light can pass the dichroic (longpass filter) and is subsequently selected for the target wavelength by the emission filter (bandpass filter, no. 4) to reduce background signal. Adapted by permission from Macmillan Publishers Ltd: (86), copyright 2005. B. Diagram depicting the light transmissions of the respective filters in A as a function of wavelength.
Conventional filter sets comprise of an excitation filter, a dichroic mirror and an emission filter. The excitation and emission filters either select for a certain bandwidth of wavelengths (bandpass filters) or allow light to pass up to a particular threshold wavelength and from a certain wavelength on, respectively (longpass filters). The dichroic mirror reflects the incoming excitation light onto the sample while the emitted sample fluorescence passes the dichroic towards the detector. Bandpass filter sets are superior to longpass filter sets as they filter out Raman scattering occurring upon laser excitation as well as can minimize further potential background signals. Longpass filter sets in turn may provide slightly higher emitted fluorescence intensity.

1.2.2 Fluorescent probes in mechanobiology

Fluorescence proteins are the gold standard to optically monitor cell architecture, such as the cytoskeleton or organelles and changes of cell organization and intracellular proteins among others. Those fluorescent studies can be conducted during live-cell imaging as well as to investigate fixated cells.

Since the identification of the green fluorescent protein back in the 1960s (104) and the unraveling of its gene sequence (105) and crystal structure (106), the use of fluorescent proteins in the natural sciences and especially biology has been established for a plethora of applications and a wide variety of organisms (84, 107-109). Fluorescent proteins are widely used in expression systems, where the fluorescent dye is linked to a particular target. This allows for the precise local assignment of proteins, the evaluation of protein compositions, monitoring of the cytoskeleton, and molecular transport within the cytosol or organelles. For example, the nanoscale architecture of integrin-based cell adhesions was identified by the expression of fluorescent proteins emitting at multiple wavelengths, each of them linked to another specific protein of the integrin transmembrane complex (110). Changes in protein conformation are commonly quantified by means of fluorescence (Förster) resonance energy transfer (FRET) assays, whereby the change in fluorescence intensity of the FRET acceptor and donor mirrors the distance of the acceptor and donor, and hence, conformational changes at the molecular level (111). The local transfer of targets can be monitored by various strategies, such as applying fluorescently tagged antibodies (112) or protein-free probes, e.g. fluorescent carbon nanotubes (113).

Since mechanotransduction processes elicit a broad range of effects commencing with architectural remodeling (114, 115) and continuing to processes involving multimodular proteins (16), which are all thought to underlie smaller and broader systemic hierarchies, the potential for the identification of these coherences with the use of fluorescence proteins is manifold. Reviews on the technological progress list this vast variety of options and strategies and should be addressed for further information (84, 108, 109). However, one particular way to directly monitor mechanotransduction processes at the molecular level is the use of fluorescent calcium indicators, which are discussed in the following section.

1.2.3 Fluorescent calcium indicators

Some fluorescent dyes change their absorption properties according to their molecular environment (101, 103). They are hence employed as sensors for alterations in this environment,
such as pH shifts or the change in concentration of a particular molecule (86, 101, 103). The fluorescent sensors are available for a broad class of molecules and the affinity towards the analyte is mostly, but not necessarily, dependent on the target cation’s degree of oxidation. To allow for simultaneous use many fluorescent sensors are available with several emission wavelengths. Due to their infinitesimal low cytotoxicity for limited time scales at low concentrations, these dyes further enable the application in biological studies.

Calcium is probably the most prevailing second messenger in biological signaling cascades (116, 117) and fluorescent sensors have been developed that undergo changes in their absorption and/or emission spectra upon calcium binding. These fluorescent calcium sensors are utilized in solution as well as in live cells when transferred into the cytosol. This transfer requires the dye to pass the cell membrane, which can either be achieved by microinjection and electroporation, respectively, or by modulating the dye so that it can diffuse passively over the lipid bilayer. For this modulation, acetoxymethyl (AM) moieties are bound to the dye making it neutral in charge thereby enabling it to overcome the cell membrane barrier (Fig. 1.8). Within the cytosol intracellular esterases cleave the AM ends rendering the dye polar. As a consequence it cannot pass the bilayer passively anymore. In this molecular state the dye is further able to bind to calcium ions, which induce its fluorescence capacity.

Fluorescent calcium indicators are very sensitive and to a great extent very selective and have been applied for monitoring of intracellular signaling cascades studying global cellular responses (118) as well as highly localized transient calcium events (sparks) (47). Though the calcium indicators serve a broad range of application, they differ significantly in terms of susceptibility to environmental factors, such as osmolarity, pH, ionic strength and protein environment among others (85). The choice of the adequate calcium sensor should therefore be carefully planned according to the experiment-specific prerequisites, which include cell species, experimental endurance, fluorescent imaging mode and many more. The major supplier for molecule-specific fluorescent dye sensors Molecular Probes (Life Technologies) provides a huge and detailed open source data and protocol collection by which experimental conditions can be evaluated during the initial study design. Additionally, excellent publications exist that not only document already demonstrated studies but same time summarize helpful advice for specific applications (119-121). Considerations for intracellular calcium measurements are discussed in the following.

Fig. 1.8: Calcium sensitive dye Fluo-4 acetoxymethylester (Fluo-4 AM, Life Technologies). The AM form of the dye is able to passively diffuse over the cell membrane into the cytosol. Intracellular esterases cleave the AM moieties enabling the dye to bind to free cytosolic calcium thereby emitting green fluorescence.
1.2.4 Considerations for intracellular calcium measurements

The quality of intracellular calcium monitoring inherently depends on (i) the experimental factors that provide optimal dye distribution within the cytosol and (ii) excitation and detection parameters, which provide fluorophore stability and clear signal to noise ratios. The following paragraphs list factors determining the final quality of cytosolic calcium measurements.

**Fluorescent dye considerations** – Calcium sensitive dyes are available as salt or acetoxyethyl (AM) derivatives. Passive loading into the cell is only possible with the AM form as already pointed out in chapter 1.2.3. The salt form of the dye can only be transferred into a cell by microinjection and is therefore rarely employed for studies of cell populations. Meanwhile, multiple calcium sensitive fluorescent indicators are available that either display an advancement of an already existing fluorophore (e.g. Fluo-3 and Fluo-4) or provide varying spectral characteristics (e.g. Calcium Green and Calcium Orange). This way the dyes can be chosen to not interfere with emission wavelengths of other fluorescent proteins already present in the sample. Advancements of the same fluorophore typically focus on adaptations in spectral distributions as well as optimizations in quantum yield and photostability (cf. www.lifetechnologies.com). The choice of a successor dye is thus commonly advantageous. Notably, different dyes show varying subcellular distributions for particular cell species. Whereas some dyes uniformly distribute within the cytosol, others undergo a higher degree of compartmentalization under identical loading conditions in the same cell species (85).

**Dye loading** – Besides the dye molecule characteristics, the dye loading procedure substantially determines the intracellular dye distribution. *(i) Detergents.* As the AM forms are hardly soluble in water, dye stock solutions are prepared in dimethyl sulfoxide (DMSO). Though dissolved in DMSO, the fluorescent molecules are still prone to agglomerate when brought into solution prior to the experiment. The addition of a detergent (0.02% pleuronic 127) supports a more equal dye distribution throughout the dye loading solution and further decreases the grade of subcellular compartmentalization (121). More detergents exist, e.g. chlorpromazine hydrochloride, that can be used to increase the dye uptake (122). However, most microfluidic devices are comprised of hydrophobic polymers, by which these agents are absorbed, and due to the very high surface to volume ratio many of these agents can hardly be administered at controlled concentrations. As the intracellular reaction cascades controlling cation influx are further prone to modulation by chemical agents, which may act as (ant)agonists to the specific cation channel as well as molecules controlling them (123), the choice of other detergents than pleuronic 127 requires detailed coordination with the study objective. *(ii) Dye administration.* Contrary to conventional cell culture protocols, dye loading on-chip requires the flushing of the dye solution into the microfluidic channels. The fluid flow-induced shear rate enhances pinocytosis (124), a specific mode of endocytosis that encloses extracellular bulk solution and molecules in lipid vesicles. These vesicles are next transported towards the endoplasmic reticulum, which results in highly compartmentalized fluorescent molecules that are inaccessible for free cytosolic calcium. The dye loading procedure on-chip has consequently to be optimized under this aspect and channel design considerations to lower the induced shear rate might be worth to be considered beforehand. Another way to limit the grade of pinocytosis is to reduce the temperature during the dye loading process from 37 °C down to 30 °C or even room temperature. Although this way pinocytosis is decreased since the lipids within the membrane become more rigid, the trans-membrane diffusion is extended at the same time. Typical dye
loading time periods range commencing at 10 minutes at 37 °C and continuing to 1 hour at room temperature. Though many protocols for various experimental scenarios exist and the dye suppliers provide basic recommendations, especially protocols for dye loading in microfluidic environments have to be tested and optimized for each and every case.

**Dye stability** – Cells loaded with a calcium indicator can principally be monitored for hours and changes in cytosolic calcium concentrations can be retrieved. Few factors however limit the time, in which the concentration of functional dye within the cytosol is constant. As a matter of fact, manufacturer’s and further literature protocols state that the experimental time should be limited to 2 hours due to the following factors (121). Though the fluorescent molecules render polar after cleavage of the AM moieties and, hence, cannot diffuse via the membrane into the extracellular space. Still, dye leakage occurs leading to a decreasing cytosolic dye concentration over time, which cannot be counteracted by adapted experimental conditions. Another factor limiting the experimental time is photobleaching induced by excitation of the fluorescent probes. By adaptation of the detection method, in particular of the mode of excitation, photobleaching can be limited but not excluded. For this, the excitation endurance and the excitation intensity can be decreased. It should be noted that the free diffusion of the fluorescent molecules and its rotational speed are retarded due to the higher viscosity of the cytosol (125, 126). This alters for example the time-dependent characteristics of photobleaching, and hence, may influence experimental outcome. The minimization of excitation endurance and intensity is therefore of high relevance.

**Fluorescence microscopy** – Transient changes in cytosolic calcium concentration may be intense, e.g. from basal 100 nM up to 1 µM (116, 120), but can also be moderate or low. Depending on the to be expected change in calcium concentration, the choice of the mode of detection is crucial. While induced calcium entry over the time scale of minutes leads to greater intracellular calcium accumulation and thus the choice of epifluorescence microscopy (wide field microscopy) serves well for the signal detection when combined with a high sensitivity camera. On the contrary, low changes as well as temporally short-termed changes (sparks) may require the application of confocal microscopy that provides much higher signal to noise ratios. Table 1.2 exemplarily enumerates few determining factors for the choice of an adequate microscopy setup for the monitoring of calcium entry under fluid flow induced shear rates.

**Tab. 1.2:** Important aspects for the choice of the microscopy setup. CM: confocal microscopy; WFM: wide field microscopy; [Ca$^{2+}$]$_i$: cytosolic calcium concentration.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in [Ca$^{2+}$]$_i$</td>
<td>CM for small $\Delta$[Ca$^{2+}$]$_i$, WFM for large $\Delta$[Ca$^{2+}$]$_i$</td>
</tr>
<tr>
<td>Global calcium increase</td>
<td>both CM and WFM</td>
</tr>
<tr>
<td>Spark detection</td>
<td>CM: stronger signal and better time resolution, limited to the detection area; WFM: critical in resolution, not limited in detection area</td>
</tr>
<tr>
<td>Magnification</td>
<td>CM: $\geq$ 40x; WFM: $\geq$ 60 x, otherwise background noise too high</td>
</tr>
<tr>
<td>Medium components</td>
<td>CM: none; WFM: exclude components with strong fluorescent properties, e.g. phenol red, to minimize background signal</td>
</tr>
</tbody>
</table>
1.3 Micro total analysis systems

Micro total analysis systems (µTAS) are integrated systems that allow all necessary steps for an analysis process in one device, i.e. sampling, sample preparation and transport, (bio)-chemical reactions, product separation and isolation, and final analysis (127, 128). The key feature of these µTAS is their small dimension, which enable e.g. better separation performance and shortened transport times for liquid and gaseous samples (128-131). For processing and analysis of liquid samples, µTAS microfluidic chips with channels are employed to miniaturize chemical and biological processes. The approach to miniaturize a larger scale process is termed downscaling (Fig. 1.9) (132). Hereby, the lateral dimensions of the system are reduced to the nano and micro-scale, respectively, which further yields room for parallelization (133).

Along with the dimensional downscaling the importance of particular process parameters shifts. In principle, the importance of a parameter depends on the order of magnitude it is calculated by. For example, the downscaling of the edge length of a cube is linear, whereas same time the volumetric downscaling of the identical cube occurs exponentially (10^3). This has considerable impact on all parameters depending on the dimensional scale, such as the number of molecules at identical concentrations and the diffusion time (cf. chapter 1.3.1). Processes that inherently rely on such parameters substantially benefit from the small sizes in terms of efficiency (e.g. better separation, higher turnover rates, higher information density), time (e.g. faster separation or faster diffusion mixing) and total sample consumption (131, 133). The examples listed in table 1.3 depict the scaling laws.

Due to the small scales prevailing in microfluidic devices, reactions can be precisely controlled. The approach to arrange molecules in an ordered fashion is termed the bottom-up approach (132). The molecular ordering can either be achieved by self-assembly, e.g. in the formation of metal organic frameworks (134, 135), or by consecutive molecular reactions, such as used in the formation of immobilized and functionalized molecular linkers (136, 137). The generation of the small dimensional environments as well as specific surface modifications requires nanofabrication methods, which are termed top-down approaches (132).

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Fig. 1.9: Top-down approach in microfluidic technology. Bulk reactions are miniaturized, set in series and parallelized. This approach allows the analysis of reactions at the micro-scale in a high throughput manner. Adapted by permission from Macmillan Publishers Ltd: (133), copyright 2006.
1.3.1 Micro-scale fluid dynamics

When fluids are handled at the micro-scale, the physical description and their behavior changes compared to the macroscopic world. In the macroscopic world inertial forces such as gravity or chaotic conditions such as turbulences dominate fluid dynamics, whereas in the microscopic scale exactly these phenomena play subordinate roles and can be neglected upon particular boundary conditions, respectively (138). The term fluid comprises liquids as well as gases and is defined as a substance, which deforms when undergoing mechanical strain. The relation of these internal friction forces to the applied strain differs for varying viscosities. At low viscosities (e.g. water) the relation is proportional and the liquid is termed a Newtonian fluid. For fluids with high viscosities, i.e. non-Newtonian fluids, the relation is unproportional. The following mathematical description of fluid flows in microfluidic environments applies for liquids that are Newtonian fluids.

Navier-Stokes equation

The description of fluid behavior in the micro-scale is dependent on dimensional parameters as well as boundary conditions. Fluid properties are characterized by the discrete quantities mass and force and as fluids are continuum materials, velocity fields underlie the continuum equation \( F = m a \). The Navier-Stokes equation depicts exactly this continuum rule per unit volume and considers linear and non-linear terms:

\[
\rho \left( \frac{\partial u}{\partial t} + u \cdot \nabla u \right) = \nabla \cdot \tilde{\sigma} + f = -\nabla p + \eta \nabla^2 u + f ,
\]

(equation 1.1)

with density \( \rho \), force density \( f \), velocity \( u \), viscous stresses \( \tilde{\sigma} \), pressure \( p \) and shear viscosity \( \eta \) over a time period \( \partial t \). The force density, or in other words, the sum of all inertial forces \( (f) \) impinging onto a fluid volume unit, is the sum of gravitational, centrifugal and electrostatic...
forces. In microfluidic environments \( f_i \) is commonly smaller than viscous forces \( (f_v) \), meaning that the non-linear term can be neglected resembling the Stokes equation:

\[
\rho \frac{\partial u}{\partial t} = -\nabla p + \eta \nabla^2 u + f.
\] (equation 1.2)

In case no electrostatic and centrifugal forces prevail, \( f_i \) can be completely neglected due to the comparably small impact of gravity. The description of the microfluidic flow in turn then requires the incompressibility of the fluid \( (\nabla \cdot u = 0) \), as the continuum equation demands the conservation of mass. The Stokes equation can hereby even be simplified further and shows that for stationary microfluidic fluid flows, for which the absence of convection can be assumed, pressure and friction forces balance each other:

\[
\nabla p = \eta \nabla^2 u.
\] (equation 1.3)

In the following dimensionless numbers are described, which provide a mathematical description of microfluidic fluid flows under these conditions as well was characterize the prevailing physical fluid characteristics in more detail.

**Reynolds number and laminar flow**

**Reynolds number**

The dimensionless Reynolds number \( (Re) \) provides the ratio of inertial and viscous forces, \( f_i \) and \( f_v \):

\[
Re = \frac{\rho ul}{\eta} = \frac{f_i}{f_v},
\] (equation 1.4)

with the fluid density \( \rho \), the fluid velocity \( u \), the dimensional length \( l \) and the fluid viscosity \( \eta \). As already mentioned viscous forces are predominant in the micro-scale, which indicates small \( Re \) numbers and the absence of turbulences (laminar flow). For microfluidic systems typical \( Re \) numbers range from \( 10^{-6} \) to 10. The critical Reynolds number \( (Re_{cr}) \) at which turbulences start to occur has been reported between 2000 and 3000 (139, 140). Flows in microfluidic devices thus clearly fall in the laminar flow regime.

**Laminar flow**

In the laminar flow regime mixing exclusively occurs by diffusion, which substantially distinguishes the microscopic from the macroscopic world. Figure 1.10 illustrates examples for laminar flows in microfluidic channels. When different fluids are brought together at a junction, they continue flowing alongside each other down the channel (Fig. 1.10A). The inter-diffusion zone spreads by diffusion only and is therefore dependent on the respective diffusion coefficient. This phenomenon can be deployed for the extraction of smaller molecules from a solution same time containing larger particles, which are not capable of diffusing as long distances as the small molecules in the identical time interval (Fig. 1.10B) (141, 142).
Fig. 1.10: Laminar flow in microfluidic devices. A Schematic drawing (left) and micrograph (right, fluorescent and bright field overlay) of a channel system into which three different solutions are introduced at equal flow rates. Fluid 3 is a fluorescein solution, which emits fluorescence when excited as shown in the micrograph. Mixing of the fluids occurs by diffusion at the fluid interfaces only, which are marked by the dotted lines in the schematic. Scale bar: 200 µm. B Schematic of the membraneless H filter (adapted from (142), with permission from Elsevier). Two different solutions are introduced from opposing sites and flown down the united channel parallel to each other. Whereas the small molecules (black dots) from the mixed dispersion are able to diffuse into the main stream of the solvent fluid due to their small diffusion coefficient, the bigger particles (red dots) stay in their carrier solution only.

Poiseuille flow and Taylor dispersion

Besides the noted boundary conditions to solve the Navier-Stokes equation, it is commonly assumed that the relative fluid velocity at the fluid-solid interface is equal to zero for stationary fluid flows (138). As a result the fluid velocity profile within the channel displays as a function of the channel cross-section and stretches into a parabola when pressure driven fluid motion is initiated (Poiseuille flow) (138, 139, 143):

\[ u_z = u_0 \left( 1 - \frac{r^2}{R^2} \right). \] (equation 1.5)

For the here depicted case of fluid flow in a tubular channel, \( u_z \) is the fluid velocity in cross-sectional direction, \( u_0 \) the velocity at \( r = 0 \) and \( R \) the radius of the tube. In case diffusion is neglected a volume unit spanning the channel (Fig. 1.11A) would thereby reveal an infinitesimally thin parabolic volume unit (Fig. 1.11B). However after a certain time, the presence of diffusion will lead to a dispersion of the molecules originally enclosed in the initial volume unit (Fig. 1.11C), i.e. the Taylor dispersion \( D_z \) (144, 145), which is defined for a tubular channel as:

\[ D_z = \frac{u_0^2 r^2}{48 D}, \] (equation 1.6)

and for a rectangular channel as:

\[ D_z = \frac{u_0^2 h^2}{120 D}. \] (equation 1.7)
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**Fig. 1.11**: Generation of the parabolic flow profile under laminar flow conditions. A At static conditions molecules in a volume unit (blue) span straight across the channel. B Upon stationary flow the molecules of the volume unit follow the flow profile as a function of the channel diameter resembling a parabolic shape. C Same case as in B same time including diffusion. This dispersion is called the Taylor dispersion.

**The Péclet number and microfluidic dispersion**

(i) **Convective mixing**

In most microfluidic applications convective transport is usually faster than diffusive transport even though dimensions are confined due to the small length scales. The influence of either of both cases is described by the Péclet number \( Pe \), which is calculated based on the fluid flow velocity \( u \), the channel cross-sectional dimension \( w \) and the molecular diffusivity \( D_m \) (139):

\[
Pe = \frac{u w}{D_m} = \frac{\text{convection}}{\text{diffusion}}.
\]

(equation 1.8)

In case of stationary fluid transport the Péclet number ranges in the dimensions \( 10 < Pe < 10^5 \). Hence, typical values for the involved variables are \( u = 10^1 - 10^3 \) \( \mu \text{m/s} \), \( w = 10^1 - 10^2 \) \( \mu \text{m} \) and \( D_m = 10^{-8} - 10^{-5} \) \( \text{cm}^2/\text{s} \).

(ii) **Diffusive mixing**

For cases in which the velocity \( u \) is extremely slow \( (Pe \ll 1) \), and hence negligible, the molecular diffusion \( D_m \) dominates mixing processes. In the special case of mere diffusion, the convective term is omitted. The diffusion time \( t_D \) is then calculated by the covered diffusion distance \( l_D \) and the diffusion coefficient \( D \):

\[
t_D = \frac{l_D^2}{2D}.
\]

(equation 1.9)

**Fluid shear forces at the liquid solid interface**

The no-slip boundary condition is a simplification of the actual physical state in stationary flow of a Newtonian fluid. In fact a finite relative velocity \( (v_{L/S}) \) at the fluid-wall interface exists, which induces a liquid-solid friction \( (\sigma_{L/S}) \) between the fluid and the wall. The friction force at this particular position can be described by the velocity \( v_{L/S} \) and the friction coefficient \( k_{FY} \) (146):

\[
\sigma_{L/S} = -k_{FY} v_{L/S}.
\]

(equation 1.10)

The slip length, i.e. the covered distance of the fluid volume at the wall interface per time interval, is hereby dependent on the fluid viscosity \( \eta \) and the wall surface roughness and hydrophobicity. The determination of the slip length is, however, complicated and it is crucial to include precise values on the involved parameters (146), which is not even possible for many
scenarios. For example, the roughness of cell surfaces has so far not been clearly defined and undergoes dynamic changes when fluid flows impart friction forces. Furthermore, although many details on the molecular composition of the cell membrane are known, precise hydrophobicity values are also not available. Due to this reason, fluid flow induced shear rates on cells adhered to the channel walls is assumed to equal the wall shear rate $\tau_{WSR}$, which is estimated without including the particular solid surface properties (111):

$$\tau_{WSR} = \frac{6 \eta Q}{wh^2}.$$  \hspace{1cm} (equation 1.11)

The estimation of the friction force considers the fluid viscosity $\eta$, the volumetric flow rate $Q$ and the channel dimensions $w$ (width) and $h$ (height).

### 1.3.2 Basic microfluidic operations

The control over fluids and reactions within microfluidic devices takes advantage from the prevailing fluid dynamic effects as well as their manipulation by microfabrication technology. The following section depicts the most commonly applied strategies to separate, mix, isolate or transfer fluid volumes within which reactions take place.

**Laminar flow** - The presence of the laminar flow regime within a channel allows the generation of (i) mixing units, (ii) concentration step profiles, and (iii) concentration gradients (Fig. 1.12A, left) (147). The diffusive width at the fluid interfaces of two joining laminar streams can be precisely tuned by the set flow velocities. However, the mixing is still limited to the molecular diffusion coefficient. To enhance the mixing at equal throughput, i.e. at an equal flow rate, microfabricated structures at the channel walls can be employed to completely mix different fluids in very short channel distances by chaotic advection (148). Controllable smooth concentration gradients perpendicular to the fluid flow direction can be created with the gradient generator (Fig. 1.12A, right). The gradient generation relies on the short merging of multiple fluid inlets (n, here: 3) and subsequent separation (n+1, here: 4) resulting in an incomplete diffusive mixing. The separated fluids are then mixed within a short staggered channel before brought into contact and the partial mixing takes place again. At the end of the mixing and re-separation unit, all channels are merged into a single channel. The generated concentration gradients are tunable by the respective fluid inlet velocities and allow for multiple gradient shapes (149).

**Valves for fluid control** - Many microfluidic devices are comprised of the polymer poly(dimethyl siloxane) (PDMS) due to its suitability for facile chip fabrication and its material properties (150-153). The introduction of polymer valves in multi-layered polymer microfluidic devices opened up a vast variety for on-chip fluid and reaction manipulation (154). Polymer valves are based on a two-layer channel network. The upper and lower channels are separated by a thin polymer membrane, which can be deformed upon pressurization of one of the channel networks (Fig. 1.12B, insert). This way not only channel constrictions can be caused, but also the complete closure of a channel so that fluid flow is either enabled or volume units are isolated. Another option that emerges from these valves is its use as pumps. Upon the consecutive actuation of multiple valves arranged in close proximity at one channel, the fluid in the channel is pneumatically pumped forward. The flexibility the polymer valves offer for fluid manipulation in one single microfluidic chip launched the concept of integrated microfluidics. Figure 1.12B
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illustrates a complex example of an integrated microfluidics device (155). Valve-based metering or mixing pumps induce fluid transport (three pumps in a row, yellow) and single valves (red) isolate fluid compartments. In addition to the complex channel networks an anion exchange column is incorporated for substrate concentration, which underlines the potential of µTAS devices.

**Droplet microfluidics** - A third strategy to handle small fluid volumes is the generation of droplets. When aqueous liquids are introduced into a continuous flow of oil (T-junction or flow-focusing), small plugs form inside the oil stream due to the immiscibility of the two fluids (Fig. 1.12C). The enclosed volumes are controllable in their size and throughput. Typical droplet sizes range from about 1 pl to approximately 100 nl and are set primarily by the channel cross-sectional dimensions and the fractions of the respective fluid flow rates (156). The plug generation frequency ranges from few droplets per second to more than 10 kHz (157). Multiple aqueous solutions can be merged prior to the plug generation to initiate reactions inside the droplets. This way time dependent reactions can be precisely monitored as the onset is equal for all volume units and consecutive analyses downstream of the droplet generation display increasing reaction times. The liquid mixing inside the droplet is hereby not merely dependent on diffusion as the volume compartment is affected by friction with the channel wall, which induces droplet-internal recirculation streams (Fig. 1.12C) (158).

For further information on microfluidic applications a vast amount of review articles exist that provide a brought overview over basic operation modes and particular developments in the areas of chemical reactions, biological quests and physical research. A few recommended reviews are listed in table 1.4. Microfluidic platforms for cell and cell content analyses are discussed in the following section in more detail. µTAS devices for mechanobiological applications are reviewed in chapter 2.

**Tab. 1.4:** Selected reviews on microfluidic technology and applications.

<table>
<thead>
<tr>
<th>Field</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>General microfluidics</td>
<td>(128-130, 159, 160)</td>
</tr>
<tr>
<td>Design and material considerations</td>
<td>(131, 132, 150, 153, 161-165)</td>
</tr>
<tr>
<td>Chemical reactions</td>
<td>(166-168)</td>
</tr>
<tr>
<td>Drug analysis</td>
<td>(133, 169, 170)</td>
</tr>
<tr>
<td>Cell / biochemical analysis</td>
<td>(164, 171-177)</td>
</tr>
<tr>
<td>Material research</td>
<td>(178, 179)</td>
</tr>
<tr>
<td>Droplet microfluidics</td>
<td>(157, 180, 181)</td>
</tr>
<tr>
<td>Analysis-based design considerations</td>
<td>(182, 183)</td>
</tr>
</tbody>
</table>
Fig. 1.12: Manipulation of fluids at the micro-scale. A The laminar flow regime is utilized to induce fluid environments with sharply separated and defined mixes of solutions in a single channel, respectively. (left) Parallel laminar flows induce varying patterns of different solutions. The mixing at the solution interfaces occurs by diffusion and can be tuned by the respective flow velocities thereby either separating the areas or inducing moderate dispersion. Reprinted with permission from (184). Copyright 2005 American Chemical Society. (right) Controlled concentration gradients of multiple shapes form when diverse liquids carrying varying concentrations of substances are introduced into the gradient generator. Reprinted with permission from (149). Copyright 2001 American Chemical Society. B The introduction of polymeric valves (left, insert) paved the way for integrated microfluidics. From (154). Reprinted with permission from AAAS. (left) Valves cannot only be applied to compartmentalize fluids (red valves) but also for fluid transport and solution mixing. Three consecutively arranged and actuated valves (yellow valves) are hereby used as pneumatic metering pumps. (right) Photograph of the microfluidic chip device bearing multiple fluid and control connectors to address the integrated channel network depicted in the micrograph, which is visualized with dye. Adapted from (155). Reprinted with permission from AAAS. C Droplet microfluidics is based on the immiscibility of aqueous solutions and oil and are frequently used when pico- or femtoliter volume units need to be enclosed and analyzed at high-throughput. (left) Different solutions can be enclosed inside a droplet, which mix after the droplet has formed. (right) Micrograph and schematic drawing of the droplet generation for droplets bearing multiple fluids. The internal mixing is reinforced by the winding channel structure, which shifts the fluid flow hemispheres within the droplet plugs. Adapted from (181). Reprinted with permission from John Wiley and Sons.
1.3.3 Microfluidic devices for cell analysis

Microfluidic devices for cell culture and (content) analyses are highly specialized for each individual case to meet the requirements for cell culture conditions of the particular species if necessary and the respective analysis objective (171, 173-176). Conditions for cell culturing on-chip and the effects of microculture on cell metabolism and biochemistry have been studied (185) and culture protocols exist for various cell species, which rely most commonly on perfusion protocols (173, 186). Cell analyses span the whole range from whole cell to content analysis with the focus on e.g. cell physiology, gene, metabolite and protein expression patterns and catalytic activity among others (187-191). The following sections briefly review different options on how cells are manipulated and (genetically) analyzed on-chip.

(Single) cell manipulation in microfluidic devices

When introduced into the microfluidic chip, fluid flow directs cells to the position where they are either left to proliferate or analysis succeeds. For the continuous monitoring at this particular location on-chip a fixed positioning is necessary to circumvent challenging tracking algorithms. Further, local positioning may be a requisite to allow for cell isolation or contained association with e.g. other cells, patterned substrates, and selective agent supply sources such as channels, pores or vesicles. A couple of methods exist that facilitate the controlled location of (single) cells, namely hydrodynamic trapping, trapping by means of optical, acoustic, magnetic and electrical tools, valve based isolation, droplet enclosure, and locally-controlled cell seeding of adherent cells (192, 193). A few of these methods are explained in more detail in the following.

Hydrodynamic trapping is maybe the most commonly applied strategy to trap cells, as besides the physical trapping structures no additional technical resources are required. The trapping hurdles are incorporated in the chip device and match the cells dimensions, so that a single cell is captured per trap (Fig. 1.13A) (194, 195). Hurdle arrays allow for the high-throughput analysis of single cells. Modifications of the hurdle geometry render more complex applications possible, such as the controlled association of two single cells for subsequent fusion (196, 197). A contactless option to isolate single cells or cell populations is the integration of valves to form enclosed volume units. The first example for a semicontinuous micro-bioreactor was introduced by Balagaddé et al. (Fig. 1.13B) (198). Integrated microfluidics applying polymer valve technology built a channel network for steady-state growth of *E. coli* cultures at controlled dilution rates and medium supply in the absence of biofilm formation. A peristaltic pump operated the culture circulation in the ring shaped microchemostat and cell washout, medium renewal and biofilm removal occurred compartment-wise to avoid elaborate complete culture transfer. Although in contrast to the physical traps this system enables the enclosure of microcultures, the isolation of single cells is intricate and not as reproducible as when using the hurdle traps. The combination of both strategies however not only allows for single cell capture but as well its isolation in picoliter volumes as illustrated in figure 1.13C (199). Due to the very small volume inside the closed valve, intracellular compounds set free from a lysed cell still remain in concentration ranges, which can be quantified by highly sensitive techniques, such as enzyme linked immunosorbent assays (ELISA) (199, 200).

Another trapping method is dielectrophoresis (DEP), which is based on the force of a non-uniform electric field exerted on a dielectric particle. All particles exhibit dielectrophoretic
activity in the presence of an electric field, even without being charged, which opens up the option to manipulate cells in solution. In negative DEP (nDEP) the dielectrophoretic force leads the cell away from areas of high field regions. Hence, a careful design employing nDEP allows contactless trapping of single cells at defined positions (Fig. 1.13D) (201). Generally, larger cells can be trapped more easily due to higher dielectrophoretic activity. However, studies have as well demonstrated the successful trapping and cultivation of small bacteria (202). Although the presented methods to manipulate, capture and isolate cells out of a cell population have so far comprised a wide variety of very useful applications, they all are limited in the local transfer of an isolated cell. Droplet microfluidics enable the isolation of cells in defined volume compartments that can be transported and analyzed (Fig. 1.13E). Cells are encapsulated within an aqueous plug, which is transported through the chip by a continuous carrier fluid flow (oil) at high frequencies (cf. chapter 1.3.2.). Combining cell suspensions with fluids containing specialized detection agents, such as antibody-labeled beads, the high-throughput detection of antibody secretion from single cells was demonstrated (203).

A fundamentally different trapping method pertains to the locally defined culture of adherent cells. Cells in suspension can be manipulated identical to particles. i.e. by mere controlled fluid handling. On the contrary, adherent cells have to attach to a substrate by necessity to develop specialized physiological characteristics. In addition, species-dependent requirements may stipulate freedom to migrate, attain cell-to-cell contacts or cell-to-cell communication by secreted factors as vital biological processes (204). Substrate functionalization is frequently chosen to provide these vital parameters as well as retain cell patterns in locally defined areas. The substrate functionalization typically comprises proteins of the extracellular matrix cells adhere to, but can also consist of coatings that prevent cell attachment (205, 206). For locally defined functionalization different options can be considered. Surface coatings can either be created using microcontact printing (205, 207) or by the use of laminar flow streams as shown in figure 1.13F (208). The parallel introduction of solutions bearing cell adhesion promoting molecules and cell repellent molecules into a single channel leads to parallel surface functionalization due to the laminar flow regime. The hereby-generated separated cell populations can further be differently stimulated, again applying laminar flow conditions.
Fig. 1.13: Microfluidic strategies to capture or isolate (single) cells for analysis. A For the hydrodynamic trapping of cells posts are implemented in the channel, by which cells are captured. Single cells can be monitored over time as their position in the flow channel is fixed. Adapted with permission from (195). Copyright 2006 American Chemical Society. B More complicated valve networks can as well comprise small culture chambers. The advantage is the precise control over the medium composition for each single culture and its compatibility with cell lysis and subsequent transfer of controlled volume units. Adapted from (198). Reprinted with permission from AAAS. C The combination of hydrodynamic cell traps and valves facilitates the isolation of cells in picoliter-sized volumes and the analysis of intracellular compounds at the single cell level. Adapted from (199) with permission of The Royal Society of Chemistry. D Negative dielectrophoresis is a useful tool to distract the flow director of cells in suspension as well as to reversibly trap cells without the necessity of direct contact. Adapted from (201) with permission of The Royal Society of Chemistry. E Droplet microfluidics are frequently chosen to isolate single cells, analyze them or secreted products and to sort them based on the previous analysis outcome, all in a high-throughput manner. Reprinted by permission from Macmillan Publishers Ltd: (203), copyright 2013. F Adherent cells can be selectively seeded in microchannels upon parallel laminar flow streams, which can same time be utilized for the selective treatment by the generation of different chemical environments. Reprinted from (208), with permission from Elsevier.
Microfluidic single cell content analysis: gene expression

Cell developments and responses to drugs are not only reflected by varying levels of metabolic and catalytic activity but often manifest in changes in gene expression. These changes may result in transcriptional changes, varying levels of protein expression, and determination on cell fate, i.e. differentiation or apoptosis (204). Commonly mean expression levels of a whole cell population are measured to access the gene expression levels. These mean values are however not capable to represent cell-to-cell differences or cell heterogeneities that can affect the future development of the whole population (177, 209). As microfluidic technology provides extraordinary performance due to low sample consumption and handling, and additionally provides high sensitivity for analyte detection when coupled with fluorescence spectroscopic methods, the demands for the analysis of single cell gene expression levels are matched. The first demonstration of microfluidic polymerase chain reaction (PCR) was realized in a continuous flow device (Fig. 1.14A). Complementary DNA (cDNA) samples were combined with a buffer mix containing specific primers and the solution was driven through a microfluidic channel that spanned over the three required temperature zones for DNA strand melting, extension and polymerase annealing (210). The final product was collected and analyzed off-chip. Complete microfluidic quantitative PCR (qPCR) at low template starting concentrations was first introduced using droplet microfluidics, which facilitated the encapsulation of cDNA, primers and probes into droplets (Fig. 1.14B). The droplets were again lead through varying temperature zones on the chip, and the probe signal was analyzed by laser induced fluorescence after each cycle (211). Although the used template concentrations matched those in a single cell, this approach still did not allow for the analysis of cell samples.

The solution permitting gene expression analysis of single cells was again realized by integrated microfluidics (Fig. 1.14C). The process sequence for reverse transcription and qPCR (RT-qPCR) of single cells comprised of a combination of hydrodynamic cell trapping using hurdles and valve-based volume isolation and transfer (212). After cell capture (1, 2ii), cells were lysed in an isolated compartment. The cell lysate was then transferred into a larger compartment (2iii), where it was simultaneously mixed with the necessary buffers and enzymes for reverse transcription (RT). After successful RT the cDNA solution was transferred into a final volume compartment (2iv) by adding respective primers and fluorescent probes. Temperature cycling was achieved by Peltier elements and optical readout of the amplification by fluorescence microscopy (3). The presented RT-qPCR script builds the basic process sequence for all state-of-the-art RT-qPCR micro-devices and provides sensitivity for mRNA concentrations correlating to even less than of a single cell (4). Adaptations to the script were introduced for multiplexed gene analysis, however, cell stimulation and gene analysis were again dissected from each other (Fig. 1.14D) (213). While cell culture and stimulation occurred in a microfluidic platform (Fig. 1.14D (1)-(3)), cell lysis and RT were conducted off-chip after cell retrieval. A second fluidic chip device was chosen for the subsequent gene expression analysis (Fig. 1.14D (4)-(5)) that enabled multi-gene targeting for each sample.
Fig. 1.14: Polymerase chain reaction in microfluidic devices. A The first demonstrated PCR reaction on chip was performed under continuous flow conditions with distinct temperature-controlled areas. The PCR product was collected off-chip for further analysis. From (210). Reprinted with permission from AAAS. B Droplet microfluidics allowed first qPCR of single cDNA copies. Reprinted with permission from (211). Copyright 2008 American Chemical Society. C The use of valves enabled the compartmentalization of single cell lysates and thereby the first RT-qPCR of single cells taken from suspension. Scale bars: 400 µm. Reprinted from (212) with permission from Proceedings of the National Academy of Sciences. D Genetic analysis of adherent cells was so far realized for small cell populations only. In addition, cells first had to be retrieved from the microfluidic platform, cell lysis and RT reaction were conducted off-chip and, finally, another chip device was chosen for qPCR. Adapted by permission from Macmillan Publishers Ltd: (213), copyright 2014.
1.4 Scope of the thesis

This thesis aimed at the development of microfluidic platforms for mechanobiological studies. As discussed before, lab-on-a-chip devices allow for cell handling, manipulation and culture as well as substantially primed the progress of spatiotemporal (single) cell analytical achievements. First, the development of a microfluidic device was required that meets the demands for cell handling, culture and mechanical stimulation (i, ii). Second, the device was optimized for the C2C12 murine cell line to analyze mechanosensitive cation channels of the Transient Receptor Potential (TRP) class (iii). Besides this in vitro investigation of mechanotransduction events, a world-to-chip interface was developed to study the effects of dynamic mechanical loading of bone on the basis of in vivo samples (iv). The thesis is structured as follows:

i. Chapter 2 discusses the latest advancements of microfluidic platforms in the field of cell mechanobiological research including technological prospects that microfabrication technologies offer. The next section lists all technical fabrication processes employed in this thesis as well as specialized chip preparation methods and cell culture in detail (chapter 3).

ii. Long-term cell culture and analysis implied the engineering of a stage perfusion incubator rendering the on-chip C2C12 culture, mechanical intervention and simultaneous optical analysis possible (chapter 4). The incubator had to ensure temperature control and pH regulation of the culture media as well as to enable mechanical fixation of the chip device including peripheral supply tubing. Further, the compatibility of the incubation system with standard inverse microscope stages had to be ensured.

iii. As outlined in the introduction, mechanotransduction processes are often mediated via cation channels of the TRP class. The developmental programs of murine myoblasts inherently depend on the functional gating of these channels, which has been studied extensively. However, these studies still bear particular drawbacks in terms of invasive stimulation and readout, and to a greater extend do not enable the concurrent and continuous monitoring of small communities of cells (cf. chapter 1.1.3). This project thus intended to tackle exactly these challenges (chapter 5). Applying fluid-flow induced shear stress and optical monitoring of the calcium entry via TRP channels facilitated non-invasive mechanical stimulation and readout. Although this mode of the mechanical stimulation is non-invasive, other “invasive” modes such as compression and tensile stretch exist that may recruit other TRP channel classes. By choosing the stimulation mode fluid shear stress, a predilection of TRP channel class was to be expected. The mechanically-mediated changes in intracellular calcium levels were determined by means of fluorescence microscopy using calcium-sensitive dyes.

iv. Chapter 6 aims at the analysis of in vivo stimulated bone samples. Mouse vertebrae were repeatedly mechanically loaded in vivo prior to sample harvesting and the preparation of cryosections. To analyze the response to the mechanical strains, a microfluidic interface was developed that enabled capturing of single cells from the bone section. The device should enable analysis of multiple genes on the single cell level and ultimately allow for the correlation of the strain energy densities to gene expression levels.
Finally, chapter 7 provides a concluding discussion and nominates future perspectives for the technical developments as well as the new biological insights acquired in this work.
Chapter 2

A new mechanobiological era: microfluidic pathways to apply and sense forces at the cellular level

Fueled by technological advances in micromanipulation methodologies, the field of mechanobiology has boomed in the last decade. Increasing needs for clinical solutions to better maintain our major mechanosensitive tissues (muscle, bone, and cartilage) with increasing age and new insights into cellular adaptations to mechanical stresses beckon for novel approaches to meet the needs of the future. In particular, the emergence of microfluidics has inspired new interdisciplinary strategies to decipher cellular mechanotransduction on the biochemical as well as macromolecular level. Cellular actuation by locally varying fluid shear can serve to accurately alter membrane surface tension as well as produce direct compressive and strain forces onto cells. Moreover, incorporating microelectronic technologies into microfluidic platforms has led to further advances in actuation and readout possibilities. In this review, we discuss the application of microfluidics to mechanobiological research with particular focus on microfluidic platforms that are able to simultaneously monitor cellular adaptation to mechanical forces and interpret biochemical mechanotransduction.

This chapter was published in:

Contributions

F. Kurth developed the review structure, selected all literature, wrote the manuscript, and created figures. K. Eyer created figures and wrote the manuscript. A. Franco-Obregon and P.S. Dittrich wrote the manuscript.
2.1 Introduction

It is no surprise that cells respond to changes in their environment. External stimuli in the form of diffusible chemical messengers are first transmitted to cells via their binding to receptors either on the cell surface or within the cytosol. Physical stimuli (e.g. temperature and pressure), on the other hand, are commonly perceived and transduced into cellular responses by cation channels of the transient receptor potential class (45). Both modes of cellular stimulation share general features: immediate responses to environmental stimuli are commonly, but not solely, initiated by changes in cytosolic calcium levels. These, in turn, modulate passive cellular properties, for example, the membrane potential, and downstream cellular responses, for example, activate second messenger cascades, regulate cellular biosynthesis and the cell cycle. Delayed responses to external stimuli evoke changes in gene expression that may, or may not, consolidate the signals to redirect cells into another developmental state. One very important class of physical stimuli with fundamental developmental consequences is the various modes of mechanical input perceived, or generated, by cells.

Mechanical forces originating from the extracellular environment are constantly being exerted upon cells and cells themselves impose counteracting forces onto their surroundings. Cells are intrinsically sensitive to the physical properties of the extracellular environment (topography and stiffness) as well as the composition of the extracellular matrix (ECM). As a result, cells also receive mechanical input from neighboring cells that are in direct contact. All tissues constantly experience dynamic and sustained exogenous mechanical forces, including the constant force of gravity that direct their developmental programs. While cells can endure mechanical strain and partly resist deformation, they are not unresponsive to mechanical input and this form of stimulus can provoke cellular responses and consequently influence or regulate cellular function and fate (20, 21, 214, 215). This can occur by two modes, namely transmission and transduction. Transmission is the manner in which mechanical forces can induce rearrangements of the cytoskeleton and associated organelles, thereby directly transmitting mechanical forces to remote regions of the cell. In mechanotransduction, mechanical forces are converted into biochemical cascades via force-sensitive ion channels or membrane proteins (3, 45). The ensuing response can then influence either cell-ECM or cell attachment, which, in turn, may influence fate decisions in yet uncommitted cells (216-218).

Mechanobiology has become a major research focus in the last few decades due to the ubiquitous impact that mechanical stresses have been shown to impart on cell behavior. The growing interest in mechanobiology has been further fueled by the increasing appreciation that most of our body mass arises from developmental programs that are regulated by mechanical input (20, 219). In particular, myogenesis is very developmentally sensitive to mechanical input (exercise) and our largest tissue skeletal muscle has evolved to play a major homeostatic role in the maintenance of our other body tissues (25, 220). Accordingly, alterations in cellular mechanotransduction with advanced age result in muscle loss and consequently increased fragility and morbidity in the elderly. Cellular mechanotransduction is also necessary for the maintenance of most differentiated tissues, such as osteoporosis (11), and is involved in the unabated proliferation of cancer cells that are unable to undergo terminal differentiation (2, 221). Mechanobiology, as a field of research, is therefore of great clinical importance, particularly in areas of tissue engineering, regenerative medicine, and geriatrics.
Microfluidic pathways to apply and sense forces

Fig. 2.1: Cells in the human body are frequently exposed to different mechanical forces. The table lists different types of mechanical forces that are discussed in this review and depicts microfluidic methods to simulate the natural situation.

The development of novel platforms that apply various modalities of mechanical stimuli has led to the identification of some of the molecular components involved in mechanotransduction. However, to fully appreciate and understand the combinatorial effects that interplay between mechanical properties and force-induced biochemical signaling, tools are required that enable both the control and manipulation of the cell microenvironment as well as monitoring of the resulting cellular response (222-224). Experimental platforms to monitor single, or small colonies of cells, in response to substrate-mediated or cell-mediated mechanical stresses are necessary to understand how cells are able to decipher subtle changes in common factors to evoke distinct developmental responses. However, current methods usually do not allow for multivariant stimulation and do not address individual cells.

2.2 Microfluidics as an enabling technology

Advancements in microsystems engineering in the last decade enabled the development of novel cell analytical platforms with unprecedented properties (171, 174, 175). Microsystems are perfectly suited for analyses of individual cells as well as more complex cell culture and tissue systems since the integrated tools match the length scales of cells (i.e. 5–50 mm) and moreover, small liquid volumes can precisely be controlled enabling very accurate definition and control of parameters in the cellular environment. Among the further ingenious features that microsystems technology offers is the integration of microsized, movable, fast-operating components, such as valves and pumps to directly guide fluids and particles as well as to define subcompartments by the de novo creation of barriers (e.g. microsized cell culture chambers) (154). Furthermore, these pneumatic or hydrodynamic modules can enable dynamic application of defined forces onto cells.
Miniaturized platforms for biomechanical research on the cellular level have been developed over the last decade (222, 225-227). In this review, we emphasize the enormous progress realized over the last two years by combining conventional cell culture strategies with microfluidic devices (Fig. 2.1). We discuss the trends for determining the influence of macroscopic forces, that is, fluid shear stress, compressive and stretching mechanical loads and gravity exposed to cells and tissues, as well as of microscopic biomechanical forces that are exerted by a cell. Importantly, these effects do not appear isolated under physiological conditions, but rather interacting and complementing one another either constantly or dynamically.

2.3 Stimulation via macroscopic forces

2.3.1 Shear stress

The laminar flow regime that is achievable with microdevices facilitates the application of well-defined flow rates and profiles within the microfluidic channels and hence, allows for the local stimulation of cells with precise shear forces. It is thus not surprising that microfluidic devices have often been developed to study the influence of shear stresses on adherent or cells in suspension. Very exciting and noteworthy are recent studies examining combinatorial effects, such as simultaneous microenvironmental altering. For example, the combination of shear stress and nanoscale surface topographies allows the study of cell–matrix interactions and cytoskeletal reorganizations in response to combined intrinsic and extrinsic forces, more closely mimicking the cellular environment inside a behaving organism (228). Nanogratings either orthogonal or in parallel to applied fluid flow in the microchannel guided cytoskeletal dynamics of human mesenchymal stem cells (hMSC) with respect to cell adhesion, morphology, and migration (Fig. 2.2a). Noteworthy in this study is the observation that these parameters influenced nuclear positioning and shape, actin cytoskeletal reorientation, and focal adhesion development in response to combined fluid flow and surface nano-topography with the aim of finding novel strategies for the regulation of more integrative cell behaviors such as proliferation and differentiation.

Clinical research strategies often focus on drug delivery, the transport of chemical agents to target tissues. The transport of solutes within microvessels and interstitial fluids depicts a dynamic system under the influence of fluid shear stresses that can be nicely simulated within a microfluidic device. An interesting example was given by Chin et al., who built a vascular mimic for the investigation of organismal-like variation in fluid shear stresses and gradients of glucose concentrations delivered to endothelial cells (Fig. 2.2b) (229). Herewith, resting conditions, that is, moderate shear stresses, and increased pulsatile surface-tension forces to simulate exhaustive exercise were successfully recapitulated. Additionally, they compared physiologically relevant glucose variations present in healthy persons and diabetic patients. The results of the study revealed a direct correlation between fluid pulsatile profile and the production of reactive oxygen species, clearly underscoring the importance of mechanotransduction processes for clinical studies.
In addition to the biomimetic systems, the study of fluid shear forces on (individual) cells and its perturbation of intracellular organization has also provided surprising results. For example, instead of an expected cytoskeletal stabilization upon increased shear stress, Rahimzadeh et al. reported cytoskeleton relaxation (111). Particularly in cell-based assays, microfluidic methods should complement routine cell-based strategies. This is nicely demonstrated by a recent work by Wang et al. who developed a microfluidic platform for cell seeding and performing multishear stress assays (230). Afterwards, the microfluidic component could be disassembled and conventional immunostaining assays performed on the cells adhered to the glass substrate.

2.3.2 Tensile stress (stretch, strain)

The integration of flexible cell-substrates into a microfluidic device has permitted studies of tensile stresses onto distinct cell types and tissues. Cells within organs such as the lungs, heart, blood vessels, and muscles are continuously exposed to highly variable strain forces due to obligatory physiological function and superimposed body movement. A novel system to study similar forms of cyclical mechanical stimulation was developed by Huh et al., who designed a microfluidic device that is able to appropriately mimic the mechanical environment of the alveolar–capillary interface of the human lung (Fig. 2.2c) (231). A thin membrane separating air-filled epithelial from the liquid-filled endothelial compartments could be stretch-activated by adjacent vacuum channels, leading to a cyclical mechanical strain on the indicated cell types. This ‘lung-on-a-chip’ model was employed to systematically study the harmful effects of bacterial infection and nanometer-sized particles on the lungs without necessitating animal experiments. It particularly revealed a correlation between cyclic mechanical strain and inflammatory responses of the lung epithelia to bacteria and silica nanoparticles intended to mimic airborne particulates. This approach offered insights into an important physiological process and suggested that the mechanical movement associated with breathing is responsible for, for example, particulate uptake into the microvascular channel system.

Another alveolar model system investigated the correlation of solid and fluid mechanical stresses, independently and in a combinatorial manner, on alveolar epithelial cells, simulating ventilator-induced lung injury (Fig. 2.2d) (232). In addition to the ‘conventional’ alveoli model, an in vitro airway system was introduced that could apply surface-tension stresses by the constant propagation of an air–liquid interface. Using this approach, significantly higher cell death and detachment rates were observed with simultaneous stimuli. The results from this study underscored the benefit of minimizing surface-tension stresses (e.g. by surfactant therapy) to reduce the severity of surface-tension pathology.

The cardiovascular system has also been the focus of several recent studies examining the effect of fluid flow over complex vascular model systems. An in vivo-like system for the study of vascular function was made available in the Endothelial Cell Culture Model (ECCM) (233). Basically, the system consists of a cell culture chamber within which a thin elastic membrane for cell attachment and mechanical actuation is implemented. Human aortic endothelial cells were seeded onto the membrane using conventional cell culture conditions. Following recovery and proliferation of the cells were challenged by various physiological flow rates and pressure profiles representative of either healthy or disease states. Lone modal stimulation or combinations of constant or pulsed fluid flow as well as various degrees of applied strain could recapitulate states of heart failure, hypertension, hypotension, tachykardia, and bradykardia. In
summary, the developed model provides a platform in which single or various combinations of mechanical stimuli can be applied and potentially, be used to predict patho-physiological outcome.

Fig. 2.2: (a) Polymer thin film technology was used to design a nanopatterned surface by a stitching technique to control cytoskeletal arrangements and manipulate focal adhesions. Fluid shear induced surface tension furthermore altered the preset cell characteristics influenced by the given nanotopographies (reprinted from Ref. (228) with permission). (b) A multilayer PDMS chip including valves for flow control, a microdiluter channel design for selective chemical treatment and a programmed syringe pump system for fluid flow modes were combined to mimic several physiological conditions characterized by varying shear stresses and glucose concentrations (reprinted from Ref. (229) with permission). (c) A two layer PDMS microchip was arranged to facilitate key structural, functional and mechanical properties of the alveolar–capillary interface, in which human alveolar epithelial cells and microvascular endothelial cells were cultured on opposing sites of a porous membrane (reprinted from Ref. (231) with permission). (d) A two layer PDMS device using a thin membrane for strain stimulation on attached epithelial cells builds up the microfluidic alveolar model by Douville et al. (reprinted from Ref. (232) with permission).
2.3.3 Cell compression

Cell types such as osteocytes and fibroblasts frequently experience compression. The ensuing deformation may have long-term developmental consequences such as promoting proliferation or producing aberrant cell responses. For instance, altered abilities of diseased cells to deform upon compression have been observed in numerous cell types (234), although with lack of throughput (235). Hence, microfluidic cytometers have been developed to identify potentially cancerous cells based on their compression responses in a high throughput manner (236). By combining a polymer cantilever-based method with microrestriction flow through, the cell’s mechanical response to physical compression could directly be measured. Further microrestriction flow through studies relied on a combined measurement of deformation and impedance profiles (237).

Paracrine release of diffusible bioactive factors in response to mechanical deformation can be discerned by following the responses of downstream communities of unstimulated cells. HL-60 cells in suspension were mechanically compressed, triggered by means of a flexible PDMS membrane (238). This device further enabled the entrapment of downstream cell communities before mechanical stimulation on upstream-trapped cells, thus allowing the investigation of cell signaling during and after mechanical stimulation.

The monitoring of real time tissue homeostasis under physical load faces certain obstacles. Besides the need for the local precision of actuation, the selected load has to be exact over an extremely small range of forces. To conquer these issues, a biomedical device for the controlled injury of axons was established using valve-based microfluidic methods (239). This platform (Fig. 2.3a) facilitated the deciphering of mechanisms for remodeling, degeneration and regrowth of primary hippocampal neurites induced by controlled injury pads.

2.3.4 Gravitational forces

The unyielding force of gravity is ever present on earth and consequently, all cells have evolved molecular mechanotransduction apparatuses that operate efficiently in a constant terrestrial gravitational field. The impact of gravity on the cellular level, however, has only been investigated in few studies thus far, mainly due to the lack of stable monitoring devices operational under the extreme conditions characteristic of microgravity platforms. Although the long-term consequences of altered gravitational force have been best analyzed in animals, the discovered aberrancies have offered significant insights into cellular mechanotransduction.

Aiming at the investigation of mechanosensitive ion channels, Schaffhauser et al. developed an extraordinary robust system, the asymmetrical transoocyte voltage clamp (ATVOC) method (Fig. 2.3b), that has permitted the noninvasive registry of mechanically regulated membrane current under varying gravity levels (7). Transmembrane measurements of ion flow through epithelial sodium channels (ENaCs) could be monitored at nanoampere resolution under conditions of microgravity and hypergravity with high reproducibility in Xenopus laevis oocytes. Given the small size of the device and peripheral equipment, the entire experimental setup can also be mounted in aircrafts undergoing parabolic flights or within centrifuges to study hyper-gravitational and hypo-gravitational effects.
Simulated microgravity can also be obtained with the use of a random positioning machine, or 3D Clinostat. Using this technology, a real time study of morphological developments in mouse myoblasts under microgravity was performed by Pache et al. (240). Merging cell culturing within a mounted flow chamber with digital holographic microscopy revealed subtle changes in cytoskeletal organization without the use of fluorescently labeled probes.

2.4 Microtechnologies to unravel cellular microscopic forces

In addition to reacting to mechanical forces, cells and tissues also exert mechanical forces. The cell-substrate interaction plays an important role in cell development and substrate characteristics can easily be altered in a 2D fashion. Recent studies examining the effects of substrate elasticity over vascular smooth muscle differentiation (241) and myoblast migration (242) have provided promising findings for mechanotransduction research. Although the engineering of biocompatible cell culture surfaces with distinct elastic moduli has already provided important insights into cell fate decisions, there is still huge space for technological progress.

Cell contractility has been under intense investigation, for example, in the areas of cell adhesion and migration (110, 243) and synchronous contraction of cardiac myocytes (244). The possibility to measure cell contractile forces, ~10 nN per focal adhesion, has now been made possible with polymeric micropost arrays made by soft lithography, whereby the deformation of pillars fabricated from well-characterized materials enables the determination of contraction forces generated by cells grown on top or in between the pillars. Fu et al. regulated pillar stiffnesses by altering their length and were able to mechanically direct stem cell fate decisions accordingly (245). Exposing cells to varying micropost rigidities also modulated cytoskeletal contractility. Combining these micropost arrays with single molecule fluorescent force spectroscopy unraveled assembly and disassembly mechanisms of focal adhesions under tension as well as allowed the calculation of the trans-vinculin force produced in stable focal adhesions to ~2.5 pN (246).

Micropost arrays have also been successfully employed in more sophisticated designs (Fig. 2.3c) as shown by Mann et al. (247). Clamping a stretchable polymeric membrane possessing a micropillar fabricated surface into a cell-culturing module facilitated the measurement of intracellular microscopic forces with dynamic extracellular macroscopy. Live-cell subcellular dynamic responses of vascular smooth muscle cells to a sustained static equibiaxial strain induced biphasic cytoskeletal reorganization: after an initial phase of cell stiffening to resist rapid cell deformation, cells adapted their architecture by a slow inelastic softening to adapt to the new microenvironmental parameters.

Finally, the integration of micropost arrays into conventional microchannel devices enabled studies investigating the impact of fluid flow induced shear stress on subcellular responses with high precision. A device with a ‘micropost array in a channel’ (mPAC) (Fig. 2.3d) allowed the detailed study of shear-mediated mechanotransduction in endothelial cells (248). This platform offers both controllable surface rigidity and label-free force sensing, while benefiting from all the advantages microfluidic platforms provide.
2.5 Future challenges and opportunities

Microfluidic platforms enable the precise spatial and temporal investigation of cellular mechanical transmission and mechanotransduction processes extending from the tissue level to the single cell-level. The studies presented in recent years demonstrate the great potential of microfluidic methods for biomechanical research. However, more detailed studies are required, particularly in the fields of firstly, cell-to-cell and cell-to-matrix interactions, secondly, cellular mechanically adaptive microscopic remodeling and signaling cascades and thirdly, characterization of the specific and general influences that macroscopic forces induce on cells.

In pursuing the goal of engineering functional artificial tissues, one of the most crucial aspects will lie in the construction of 3D cell culture matrices. In this regard, the successful adaptation of
biocompatible hydrogels such as poly(ethylene glycol)-based, matrigel, and collagen has advanced significantly in recent years. The precise elastic modulus gradients and the time-dependent properties can be generated and implemented in microfluidic channels (249-252). Furthermore, micromanipulation technologies employed on artificial biomembrane models, such as lipid vesicles, can help to unlock molecular organizations of biological membranes under mechanical strains (253, 254).

Microfluidic platforms already provide smart tools to achieve these goals for biomechanical research and certainly, the needs of mechanobiological sciences will further inspire technological advances. However, progress in all aspects, including surface chemistry, cell biology assays, and (label-free) analytical methods are needed. Hence, close cooperation between biomechanic researchers, engineers, and chemists is required to effectively apply and optimize these promising methods.

Acknowledgement

Funding from the European Research Council (ERC Starting Grant no. 203428) is gratefully acknowledged.
Chapter 3

Methods

This chapter lists the used materials and describes all general methods applied throughout this thesis. The methods comprise microfabrication of the master molds, soft lithography for device prototyping, microfluidic device operation, channel surface modifications, microscopy and cell culture. A table listing all materials used is available in the appendix (A.1).

Special contributions and acknowledgements

Fabrications of the master molds were conducted by P. Kuhn, B. Cvetković, B. Sebastian, and P. Verboket. The development of the fabrication process for the two-layered master molds comprising of two different photoresists by P. Kuhn is highly acknowledged. All SEM images were taken by P. Kuhn.
3.1 Introduction to microfabrication

The combination of photo- and soft lithography enables the rapid prototyping of polymer microfluidic devices (150, 151, 153). Hereby, photolithography is applied to transfer a channel design onto a master mold, from which high numbers of polymer devices can be reproduced by soft lithography at low costs. Although other prototyping technologies allow the fabrication of microfluidic chips from hard polymers, hydrogels, silicon or glass (128, 130, 162, 163), devices in the scientific community are predominantly fabricated from the soft polymer poly(dimethyl siloxane) (PDMS) (152, 255). PDMS facilitates very cheap, fast and simple production of single-use chips that can be utilized for many applications. Notably, PDMS has recently also proven to bear disadvantages for biochemical and biological aspects, particularly cell culture (165, 256), however, no adequate substitute with similar material properties has been introduced to date. Since only PDMS microfluidic devices were used in this thesis, the following sections describe their production including design planning, microfabrication of the master molds and polymer prototyping of the devices.

The basis for a new microfluidic device is to design a channel network for the desired application. The new channel system is created to scale using computer-aided design (CAD) software (here: AutoCAD, Autodesk, CA). This technical drawing is then transferred into a photomask for the production of the master mold. Different masks exist that facilitate varying levels of resolution of the master mold. Whereas e.g. conventionally used film masks allow reproducible resolutions on the master mold down to 5 µm, chrome masks enable to size this resolution down to the single micrometer scale (200). The here used masks are transparent polymer photomasks, onto which a high-resolution printer prints the channel network with opaque ink. The photomask is then employed to translate the channel system onto a substrate (silicon wafer) with a photosensitive polymer (photoresist) that after processing displays a negative imprint of the channel structures. This master mold can afterwards be repeatedly used for the fabrication of PDMS devices. The different process steps from design layout to the final microfluidic device are exemplarily illustrated in figure 3.1. All process details for the fabrication of the master mold and chip devices are provided in the following chapters.

Fig. 3.1: Design and production steps necessary for the fabrication of PDMS microfluidic devices: (a) Channel network layout (CAD software). (b) Detail of a foil photomask. (c) Photograph of a processed silicon wafer including channel systems. Photoresist feature details (insert) were imaged by scanning electron microscopy (SEM; scale bar: 200 µm). (d) Photograph of a final PDMS device equipped with a fluid reservoir. The channels are filled with dye for visualization.
3.2 Fabrication of rectangular and round shaped channel master molds

PDMS microfluidic chip devices with rectangular and round shaped channels were applied in this thesis. All master molds were fabricated onto 4” silicon wafers by photolithography in the ETH cleanroom facilities. Positive or negative transparency photomasks (Circuitgraphics or Micro Lithography Services, UK) were used for photolithography. A schematic overview of the microfabrication process is illustrated in figure 3.2.

The most commonly applied rectangular channels were fabricated using the negative photoresist SU-8. For the generation of different resist thicknesses SU-8 is available in varying viscosities, which allows the generation from very thin (about 1 µm) to very thick (about 500 µm) layers. SU-8 2015 was used for the generation of 20 µm thick layers due to its lower viscosity compared to SU-8 2050, which was chosen for larger channel heights. The following protocol describes the fabrication of a 40 µm thick SU-8 resist layer onto a silicon wafer. For the fabrication of resist layers in different layer thicknesses used throughout this thesis, the individual process steps have to be adjusted. All parameters (spin coating frequency, baking and exposure times) are listed in table 3.1.

First, the wafer was dehydrated for 5 minutes at 180 °C on a hotplate. SU-8 2050 (MicroChem, Newton, MA), was spin coated at 3250 rpm to a thickness of 40 µm. Next, a two-step soft bake was conducted on hot plates prior to the exposure of the wafer to UV light at 160 mJ/cm² through a negative foil mask at 365 nm on a mask aligner (MA-6 mask aligner, Karl Suess, Germany). The wafers were developed with SU-8 developer after a two-step post-exposure bake. Finally, the wafers were finished with a hard bake at 200 °C for 2 hours to fix the resist structures followed by silanization with 1H,1H,2H,2H-perfluorodecyl-dimethylchlorosilane (ABCR) to prevent irreversible sticking of PDMS on the structures.

The generation of round shaped channels in principle includes the same processing steps as the fabrication of rectangular molds. However, a different photoresist (AZ resist) is chosen to be able to deform the processed rectangular features into curved geometries prior to the hard bake of the wafer. The fabrication process is illustrated in figure 3.2. The fabrication details for round channels with a final height of 12 µm are described in the following. All parameters for a 12 µm high round channel and for other used heights are listed in table 3.2.

The silicon wafer was dehydrated for 5 minutes at 180 °C. Next, a thin layer of hexamethyldisiloxane (HMDS; Merck, Germany) (1 ml) was spun onto the wafer at 7500 rpm for 30 seconds to increase the adhesion of the AZ resist onto the silicon wafer. The AZ 9260 resist (MicroChem, Newton, MA) was spin coated onto the silanized wafer at 1900 rpm. Followed by a soft bake at 110 °C on a hot plate, the wafer was exposed at 750 mJ/cm² at 365 nm on a mask aligner (MA-6 mask aligner, Karl Suess, Germany) through a positive foil mask. After the development with AZ developer a reflow bake was conducted at 120 °C for 2 minutes followed by a final hard bake for 2 hours at 200 °C. Final silanization was realized over night using 1H,1H,2H,2H-perfluorodecyl-dimethylchloro-silane.
Fig. 3.2: Fabrication of master molds for rectangular and round channels using negative and positive photoresists, respectively. A transparency photomask with bare areas displaying the channels is used for the negative photoresist, which remains on the silicon wafer when exposed. For the positive photoresist an inverse version of the transparency photomask is applied, on which the channel systems are printed. The exposed regions of the positive resist are removed during development. A reflow bake is additionally included prior to the hard bake to generate the round shaped channel geometry. All sizes are not to scale.

Tab. 3.1: Processing parameters for the fabrication of SU-8 photoresist master molds used in this thesis. The final hard bake was always 2 hours at 200 °C.
Tab. 3.2: Processing parameters for the fabrication of AZ photoresist master molds used in this thesis. Not included is the HMDS pre-coating and the hard bake (for details refer to the text).

<table>
<thead>
<tr>
<th>Height [µm]</th>
<th>Spin Coating [rpm]</th>
<th>Soft bake [s]</th>
<th>Exposure (365 nm) [mJ/cm²]</th>
<th>Development [s]</th>
<th>Reflow bake [s]</th>
<th>110 °C</th>
<th>120 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>9260</td>
<td>3000</td>
<td>150</td>
<td>575</td>
<td>240</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>9260</td>
<td>1900</td>
<td>200</td>
<td>750</td>
<td>240</td>
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<td>9260</td>
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<td>360</td>
<td>876</td>
<td>240</td>
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</table>

### 3.3 Fabrication of multi-layered master molds

For certain applications, specialized channel geometries are required. For example, the channel design may demand optimization for lower surface to volume ratios to minimize analyte-surface interactions as well as the minimization of dead volumes in the supply channels to minimize necessary total reagent volume (212, 257). For these cases small supply channels combined with larger volume containing reaction chambers are advantageous. In principle, channel networks comprising of features with varying heights can be separately fabricated and aligned on top of each other after PDMS curing. This however limits alignment accuracy and induces potential leakage in between the layers. Hence, for those specialized applications master molds with several photoresist layers were fabricated enabling a one-step prototype molding.

To create multi-layered master molds, different resist layers are generated in consecutive order while the lowest features are created first and the highest features last. The following protocol describes the fabrication of a two-layered master mold containing high chambers and low interconnecting channels (Fig. 3.3). First a 12 µm high round channel network is created with AZ 9260 based on the protocol in chapter 3.2. After a shortened hard bake of 10 minutes, 100 µm high chambers are fabricated on top of the first layer using SU-8 2050. All processing parameters are listed in table 3.1 and 3.2 in detail. The resists are then hard baked for 2 hours at 200 °C and finalized by silanization over night as previously described for all master molds.

Notably, initial spin coating of HMDS onto the silicon wafer is not recommended when the second resist layer is comprised of SU-8. Using HMDS to improve the adhesiveness of the AZ resist onto the silicon wafer will negatively influence SU-8 adhesiveness. For those cases the initial silanization is either omitted or a thin (few micrometers) even base layer of SU-8 is processed onto the wafer before the AZ channel network is superimposed on top.

Alternatively, the first resist layer can also be constituted of rectangular channels and other features (e.g. pillars or blocks) with a lower height than the second layer, respectively. Also, the different features do not need to be connected or applied evenly over the whole silicon wafer. In case combined resist layers are planned to be fabricated on top of each other, the layer on top of an already created layer needs to be tuned to its partial height measured from the top of the previous layer and not the corresponding total height measured from the silicon wafer. To allow for precise alignment of a second layer onto the first layer alignment marks are required.
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Fig. 3.3: The fabrication of master molds with channel networks built by two different geometries and heights of photoresists on a single silicon wafer is conducted in two steps. First, the channel system displaying round channels is fabricated, however the final hard bake is shortened to 10 minutes. In a second step, the silicon wafer passes through another microfabrication, in which the next photoresist layer is created using a second photomask. All sizes are not to scale.

3.4 Design considerations for multi-layered master molds

The formability of the AZ resist changes the final dimensions of the features. Table 3.3 lists desired and actual channel heights before the reflow and after the hard bake. Heights were measured with a step profiler (Tencor Alpha-Step 500, Tencor, CA, 12.5 µm tip). Besides the discrepancy of desired and actual height, the 7 µm high channels showed a non-regular geometry, i.e. the edges were higher than the center of the channel. The listed values for this resist thickness are thus displayed as mean values of channel center height and channel side height. This fact is especially important, if a microchannel with precise volume or valve function is required.

The dimensional shrinking has to be taken into account when channel designs comprise of multiple photoresist layers that depend on structure overlay as illustrated in figure 3.4a. In addition to the partial shrinkage in height of the AZ resist (layer 1) during the baking steps, the length and width can loose up to 3% of their original size. This can lead to separated features, which in turn leads to channel systems that are not connected anymore when molded in PDMS. Figure 3.4b illustrates this complicacy for a double-layered master mold built of a thin AZ resist layer (channel) and a thick SU-8 layer (chamber). The overlap of the two structures was estimated to be sufficient with 10 µm to allow for continuous fluid connection. However, despite
accurate alignment during wafer processing, the different features remained separated so that fluid connection was prevented in the final polymer mold. Increasing the design overlap to 100 µm facilitated connected channel networks.

**Tab. 3.3:** Reflow and hard bake of the AZ-9260 photoresist lead to dimensional inaccuracy. Listed are the three different heights and their respective fabrication outcome tested for channels of 100 µm width. All values comprise mean values ± SD of three independent measurements.

<table>
<thead>
<tr>
<th>Set height [µm]</th>
<th>Height after re-flow bake [µm]</th>
<th>Height after hard bake [µm]</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>8.68 ± 0.23</td>
<td>8.04 ± 0.20</td>
</tr>
<tr>
<td>12</td>
<td>11.01 ± 0.70</td>
<td>10.82 ± 0.12</td>
</tr>
<tr>
<td>20</td>
<td>14.47 ± 1.00</td>
<td>12.61 ± 0.16</td>
</tr>
</tbody>
</table>

**Fig. 3.4:** (a) When master molds with multiple photoresist processing steps are fabricated, the designs have to be carefully planned. Especially round channels comprised of AZ photoresist undergo partial shrinking during the re-flow and hard bake and require sufficient design overlap. (b) In case the design overlap only adds up to 10 µm, partial shrinking of the AZ photoresist can lead to the separation of the particular photoresist layers despite precise alignment as shown in the scanning electron microscope image (indicated by white arrows). A recommended design overlay should thus add up to about 100 µm for similar dimensions. Dimensions in (b): round flat channel (AZ-9260, 100 µm width, 12 µm height); square photoresist block (SU-8, 100 µm height, width out of image).
3.5 Soft lithography for PDMS chip fabrication

Rapid prototyping of PDMS-based chip devices is based on soft lithography (150, 151, 255, 258). All geometries that are fabricated as a master mold are imprinted into the polymer. For microfluidic channels fabricated with PDMS a limiting factor is the width to height aspect ratio, which should not exceed 10 : 1 (width : height) as the channel is likely to collapse above this value. For higher aspect ratios columns have to be implemented to prevent device failure. For further information on design studies, applications and customized solutions of PDMS microfluidic devices, please refer to reviews on micro-contact printing (205, 208), biology and biochemistry (153, 164), single cell analysis (174, 176), mechanobiology (222, 259) and drug discovery (133) amongst others.

In this thesis PDMS planar microfluidic devices and multi-layered channel networks were applied. The following chapters describe their fabrication processes in detail.

3.5.1 Single layer devices

For single layer devices the polymer chip was casted from the master mold in one fabrication step. PDMS oligomer and curing agent (Sylgard 184, Dow Corning) were mixed thoroughly at a ratio of 10:1 (w/w) and degassed in a desiccator at 50 mbar for approximately 45 minutes. The polymer mixture was then poured over the wafer to the desired chip device height and cured at 80 °C for 2 hours. The polymer slab was peeled off the wafer after curing and the devices were diced to their final size. Access holes for fluid supply were punched using biopsy punchers (Miltex) at 1 or 1.5 mm diameter. The chip fabrication process is further illustrated in figure 3.5a (chapter 3.5.2).

Chips were bonded to glass cover slips (Menzel-Gläser #1 or #5, Germany) after a plasma activation of both surfaces for 45 seconds at 0.7 mbar and maximum intensity of the plasma cleaner (PDC-32 G, Harrick, NY). The bonding was finalized by storage at 80 °C for 10 minutes. Bonded chip devices were directly used for experiments if not specified otherwise. In case the devices were stored prior to bonding, they were stored in a closed box to prevent deposition of dirt or sealed with adhesive tape (Scotch Magic 810, 3M, Switzerland). Although it is generally accepted in the microfluidic community that adhesive tape shows excellent biocompatibility as it for instance hardly leaves any residues (no references available), previous studies showed that the use of adhesive tape might negatively influence the culture of mammalian cells (personal communication, Dr. Franco-Obregón). Due to this reason, the use of scotch tape was omitted for chip devices applied in biological experiments.

3.5.2 Double-layered devices for cell culturing

For applications in which the microchannel geometry requires advanced fluid control two-layer devices are used (134, 260). A thin PDMS membrane that can be deformed upon pressurization of the channel system separates these two layers from each other. Using this channel architecture the fluid flow inside a channel can be stopped completely or microsized compartments can be isolated from the rest of the microchannel environment upon pressurization of the so-called actuating or control layer (valve function) (135, 154, 199, 261). In this thesis double-layered
microfluidic chips were mainly applied for the local capture and seeding of cells inside cultivation chambers (chapter 4 and 5). Further, channel networks with valve function were established for the development of a PCR-chip device for the analysis of *in vivo* bone cell samples (chapter 6). The CAD designs of all used channel systems are available in the appendix (A.3).

The generation of multi-layered PDMS devices can be achieved by separate fabrication of the layers and final assembly by plasma bonding (137). This fabrication guarantees a very stable bonding in between the respective PDMS layers, which can withstand pressures of up to 4 bar. However, the alignment during layer assembly may be difficult as due to the plasma activation the PDMS layers stick irreversibly to each other when brought into contact. For designs, in which the alignment during assembly demands high accuracy, another strategy based on partial curing of the PDMS, layer assembly and final curing of the whole device can be applied. This way the respective layers can be brought into contact with each other reversibly: the device can be disassembled for new alignment if necessary. As in this thesis the latter fabrication process was mainly used, the next paragraphs, tables and illustrations specify this strategy in detail.

The PDMS layer bonding is based on the use of different oligomer to curing agent ratios (Tab. 3.4 and Fig. 3.5). The thick top layer was comprised of a 5:1 (oligomer : curing agent) ratio whereas the thin bottom layer was prepared from a 20:1 ratio. Both layers were created in parallel. The top layer (control layer) was fabricated by pouring the 5:1 ratio over a wafer followed by thorough degassing and pre-curing at 80 °C for 30 minutes. The device top parts were diced out of the resulting PDMS slab and channel access holes were punched with a 1 mm diameter biopsy puncher. The bottom layer (cell culture or fluid layer) was prepared by spin coating a 20:1 ratio silicone mixture onto the wafer at 1400 rpm for 60 seconds under nitrogen atmosphere (WS-400 Lite Series Spin Processor, Laurell Technologies, North Wales, PA). This spin coating frequency results in a total PDMS height of about 55 µm, i.e. the 40 µm high channel network is coated with a 15 µm thick polymer membrane. The correlation between spin coating frequency and silicone thickness is reported in detail in Schneider et al. (262). The thin PDMS layer on the wafer was pre-cured at 80 °C for 15 minutes. The two layers were then aligned and assembled under a microscope (Multizoom AZ100M, Nikon, Switzerland). Since the two layers were brought into contact in a pre-cured state, curing agent molecules were able to diffuse from the top layer (higher concentration) into the bottom layer (lower concentration) during the following final curing at 80 °C over night. Afterwards, the bonded layers were peeled off the wafer and the fluid access holes were punched (1.5 mm diameter). To guarantee reliable bonding, the instructions for the time periods in the protocol (Tab. 3.4) have to be followed. In case of higher room temperature in the laboratory (>23 °C) the pre-curing intervals at 80 °C may need to be shortened in the range of 1 to 3 minutes.
**Tab. 3.4**: Protocol for the fabrication of double-layered PDMS chip devices applying diffusion-based bonding. The assembled device can be stored at room temperature prior to final curing to promote curing agent diffusion into the bottom layer. The bonding of the layers is fortified this way.

<table>
<thead>
<tr>
<th>Time [h : min]</th>
<th>Task</th>
</tr>
</thead>
<tbody>
<tr>
<td>01:15</td>
<td>Mix 5:1 PDMS (55 g needed for square petri dish minimum) and place in desiccator.</td>
</tr>
<tr>
<td>01:00</td>
<td>Mix 20:1 PDMS (10 g) and place in desiccator. Pour 5:1 silicone ratio onto the wafer placed inside the square petri dish including a frame (polytetrafluoroethylene) to minimize required PDMS volume. Place in desiccator.</td>
</tr>
<tr>
<td>00:30</td>
<td>Transfer 5:1 PDMS to 80 °C, check if PDMS is thoroughly degassed. Start spin-coating 20:1 PDMS onto the wafer(s). Time the spin coating to be finished at 00:15 of the overall time schedule.</td>
</tr>
<tr>
<td>00:15</td>
<td>Place the spin coated wafer(s) at 80 °C.</td>
</tr>
<tr>
<td>00:00</td>
<td>Take both out of the oven. Wear gloves and work under a clean bench. Dice the top parts with a sharp blade and punch fluid access holes (biopsy punchers). Optionally remove debris with adhesive tape. Assemble both layers under the microscope. Deposit remaining viscous PDMS around the chips to facilitate removal of the membrane from the wafer after curing. Store at 80 °C over night.</td>
</tr>
<tr>
<td>next day</td>
<td>Remove from oven, peel off wafer, cut chips from the slab and punch holes for fluid access in the bottom layer. Optionally bond to glass cover slip or store in closed container.</td>
</tr>
</tbody>
</table>
Methods

Fig. 3.5: PDMS molding of double-layered devices. (a) For the fabrication of the top layer PDMS is poured over the wafer and degassed at room temperature. After pre-curing of the PDMS, the device blocks are diced out of the polymer slab and channel access holes are punched. (b) The bottom layer is created by spin coating of degassed PDMS onto the wafer to the desired height (channel + 10 µm membrane height) and a pre-curing step. Step (a) and (b) are timed to be finished simultaneously. (c) The finalized top layer device blocks are aligned onto the bottom layer and the PDMS is fully cured over night. The polymer structures can be peeled off the wafer and after punching the channel access holes for the bottom layer, the final device can be bonded onto a glass slide. All sizes are not to scale.

3.5.3 Multi-layered devices for the analysis of in vivo cell samples

The fabrication of multi-layered (3 layers) PDMS devices for the analysis of samples harvested from tissue sections required the insertion of multiple samples in a well chip design. For subsequent lysis and following analysis samples were collected in an open well chip, which was afterwards assembled onto a control layer. Fabrication of the particular layers was done according to the previously described protocols. All layers were fully cured prior to subsequent treatment. A special chip processing strategy was developed to match these demands (Fig. 3.6).
Fig. 3.6: Chip production for the PCR analysis of \textit{in vivo} bone samples. As the chip layer assembly was done after the sample insertion, sample protection during plasma activation was crucial to preserve RNA content. Although most parts of the device were plasma bonded, the whole device was additionally fixed in a clamp to prevent leakage. All sizes are not to scale.

Although different chip fabrication strategies were tested to facilitate sample loading into a not completely finalized chip device, the depicted process proved to be most reliable in terms of chip stability and alignment accuracy. Further fabrication processes were based on PDMS layer transfer using curing inhibition layers that stick to the PDMS surface (263-265). These transfer layers were comprised of polycarbonate (PC) sheets that were surface modified with polyvinyl alcohol (PVA, Sigma, Switzerland). Next, the curing agent inhibitor (Pt-inhibitor) aminoethylaminopropyltrimethoxysilane (AEAPS; Sigma, Switzerland) and/or the non-inhibiting silane 3-methacyloxypropyltrimethoxysilane (MEMO; Sigma, Switzerland) were spin coated onto the modified PC sheets. Though this processing resulted in a finalized device even before the samples were introduced, the fabrication proved to be unreliable.

Simpler chip designs for the verification of intact RNA material in the sample after laser capture microdissection were created by bonding the fluid layer directly onto a glass cover slip.
3.5.4 PDMS grid patterns

For the establishment of a world-to-chip interface samples were dissected out of a tissue section and catapulted into a well placed in close proximity above the specimen using laser capture microdissection (LCM) (chapter 6). Since the flight trajectories during catapulting from the specimen into the well never resembled each other, scattering profiles of the samples after catapulting were imposed. PDMS surfaces with imprinted square frames served as a measure for the deviation from the aimed for local target. These grid patterns were fabricated by pouring a 10:1 (oligomer to hardener) silicone mixture into the caps of 500 µl eppendorf vials. The filled caps were then placed upside down onto a heated (80 °C) wafer bearing the grid master molds (50 µm height) to cure the PDMS. The side lengths of the squared grid patterns were 100, 200, 300, 400 and 500 µm. The finalized caps were then fixed into a holder of the LCM microscope.

3.6 Microfluidic device operation

Fluids in microchannels can be manipulated by different modes of actuation, such as syringe pumps, pressure gradients, capillary forces, electric and magnetic fields, rotation, and acoustic streaming (129, 143). Syringe pumps are most commonly chosen to drive fluids through microfluidic devices as they do not require elaborate peripherals and allow different modes of actuation. Hereby, fluids can either be pushed through the channels (210), pumped in a withdrawal mode towards the syringe (266), or even actuated at in- and outlet simultaneously while one pump pushes the liquid into the microchannels and the other pump withdraws the liquid out of the chip (267). The last option can explicitly be applied when flow disturbances have to be minimized. Pressure gradients for the manipulation of liquids in microchannels are frequently derived applying a gas pressure at the device inlet. Pressure driven fluid flow is advantageous over syringe pump actuated flow in terms of fluid flow stability at low flow speeds, i.e. it is fluctuation-free. Same as syringe pumps, pneumatic controls can be programmed and actuated automatically, e.g. by solenoid actuators (212). In this thesis fluid control was realized with syringe pumps as well as with air-pressure driven pumps, which is explained in more detail in the two following sections.

3.6.1 Fluid control using syringe pump systems

All experiments performed with syringe pumps (Nemesys, Cetoni, Germany) were conducted in the withdrawal mode. Hereby, the microfluidic device was equipped with a custom-built reservoir into which the fluid solutions were pipetted (cf. chapter 4). As the experiments did not require extremely fast fluid exchange or even parallel laminar flow streams generated within one channel, this fluid control option was sufficient to comply with the particular requirements. Figure 3.7 illustrates the consecutive supply of different fluids to the microfluidic chip. By using glass syringes (Agilent Technologies, Switzerland), air-tight tubing (fluorinated ethylene propylene, BGB Analytics, Switzerland) and air-tight connections (polyetheretherketone, Ercatec AG, Switzerland) highly controlled fluid flows could be achieved (cf. chapter 5).


Fig. 3.7: Syringe pump control. The schematic on the fluid actuation in the withdrawal mode using a reservoir as fluid feed displays an iterative fluid exchange (left to right). This way surface modifications, cell supply, perfusion culture and fluid flow stimulation of cells can be conducted without the need for multiple syringes or tubing supply with integrated valves. All sizes are not to scale. Right: Photograph of the chip device equipped with the reservoir and tubing.

### 3.6.2 Pressure driven fluid flow

The filling of PDMS microfluidic devices with dead end channels is best accomplished with gas pressure driven fluid flow as the enclosed air within the channel can be forced out through the porous PDMS matrix. Hereby, the sustained pressure does not induce device failure as soon as the dead end channels are completely filled. Syringe pumps have to be stopped accurately otherwise either the chip bonding breaks or tubing connections start to leak. Notably, valve-controlled microfluidic networks work best with pressure driven fluid flow as the pressures of the control layer and the fluid layer can be tuned so that the valve control is actuated with higher pressure than the fluid control. Complicated fluid control programs can be circumvented this way: the pressure on the fluid layer is sustained permanently and flow control is derived by valve actuation only.

A custom-built pressure control device was applied to regulate nitrogen pressure in the chip device. Control layers (valves) were actuated at 2.5 bar and fluid flow was driven by 1.0 bar. Figure 3.8 shows the working principle of fluid actuation by the induced pressure gradient. Whereas the pressure in the fluid layer is kept constant, actuated and non-actuated valves control the fluid flow within the fluid layer.

### 3.7 Surface modifications

Due to the large surface to volume ratio prevalent in microfluidic devices, surface properties have to be considered. If the experimental conditions require particular surface properties, surface modifications are used (268, 269). Principally, surfaces can either be coated with molecules to block the adhesion of analytes (270) or functionalized to provide binding sites for various applications including molecules (137), vesicles (266), bacteria and viruses (136) among others. Further, surface functionalization is frequently used for cell culture applications (173, 271). In this thesis the channel surfaces were modified for cell culturing as well as to prevent analytes from adsorption to the channel walls.
Methods

3.7 Functionalization for cell culture applications

For cell culturing on chip the channel surfaces were functionalized with either fibronectin or laminin (both Life Technologies) to provide the basis for cell to surface interaction of adhesive cells. To guarantee a clean glass substrate for the cells to adhere to, glass cover slips were cleaned by sonication in ethanol (analytical grade, Fluka, Switzerland) for at least 30 minutes prior to chip bonding. A protein (fibronectin or laminin) in phosphate buffered saline (PBS) solution (0.1 mg/ml) was flushed through the channels directly before cell seeding at 37 °C for 45 minutes at low flow rates (≤ 0.5 µl/min).

3.7.2 Functionalization for surface blocking

For the development of the qRT-PCR on chip bovine serum albumin (BSA fraction V; Sigma, Switzerland), polysorbate 20 (Tween 20; Sigma, Switzerland), polyvinylpyrrolidone (PVP; Sigma, Switzerland) or poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG; SuSoS AG, Dübendorf, Switzerland) were tested to prevent sample loss due to analyte adhesion to the channel surfaces. PLL-g-PEG was flushed into the fluid layer by centrifugation at 0.002% (w/v), allowed to settle for 30 minutes at room temperature and remaining solution was subsequently evaporated at 80 °C. Since the chip PCR protocol demanded empty connection channels and reagent chambers before filling and the other surfactants are only stable in solution, all other surface modifications were not conducted prior to sample addition. Therefore, BSA (0.5 mg/ml), Tween 20 (0.1% (w/v)) and PVP (2% (w/v)) were directly added to the buffers in use.

Fig. 3.8: Flow control with integrated valves and air-pressure driven pumps. The partially false colored micrographs illustrate the working principle of fluid actuation by pressure gradients (nitrogen pressure, left to right). Valve actuation lines (red; control layer; 2.5 bar) are closed when indicated with a cross. By multiplexed valve control different fluids (green, cyan; fluid layer; 1 bar) are pushed through the channels. Interconnecting channels in between the rectangular and squared chambers are comprised of round channels enabling tight valve closing.
Tab. 3.5: Filter cube assemblies for the selection of excitation and emission wavelengths during fluorescence microscopy. ex.: excitation, em.: emission

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>UV ex., blue em.</td>
<td>360 / 40</td>
<td>412</td>
<td>460 / 50</td>
<td>Hoechst 33258</td>
</tr>
<tr>
<td>blue ex., green em.</td>
<td>470 / 40</td>
<td>500</td>
<td>525 / 50</td>
<td>Fluo-4, fluorescein, F-8888 component D beads</td>
</tr>
<tr>
<td>green ex., orange em.</td>
<td>546 / 12</td>
<td>560</td>
<td>607 / 80</td>
<td>R18</td>
</tr>
</tbody>
</table>

3.8 Microscopy

All microscopy was conducted with an inverse microscope setup (IX70 or IX71, Olympus, Germany) unless specified otherwise. When working at these microscopes, bright field images as well as wide field fluorescent images (epifluorescent images) were taken with an electron multiplying charged-coupled device (EM-CCD) camera (iXon or iXon Ultra, Andor Technologies, Ireland) at 8 or 16-bit grey scale. The source of the excitation light for fluorescence imaging was either a mercury lamp (U-RFL-T, Olympus, Germany) or a halide arc lamp (X-Cite 120 PCQ, Lumen Dynamics, Canada). The appropriate wavelengths were selected using bandpass and dichroic filters (AHF Analysentechnik, Germany; Tab. 3.5). The excitation intensity was decreased for particular tasks by a neutral density filter (12%, Olympus, Germany) and down-regulation of the excitation light source (X-Cite 120 PCQ lamp only).

3.9 Cell culture

Two different types of mammalian cells were kept in culture and used for experiments on chip. C2C12 murine myoblasts were cultured on chip for the determination of growth rates (chapter 4) and mechanical stimulation under varying conditions (chapter 5). Human embryonic kidney cells (HEK-293) were grown on-chip for long term culturing (chapter 4). Conventional cell culture (using culture flasks) was conducted in a cell culture incubator (MCO-15AC, Sanyo, labtec-services, Switzerland) at 37 °C and 7% CO₂ in a humidified atmosphere. All on-chip cell culture was conducted in a custom-built stage incubation system (chapter 4) under identical conditions. The following paragraphs provide the culture conditions and medium compositions for conventional and on-chip culture.

C2C12 murine myoblasts (ATCC, LGC Standards, France) were kept at low confluences to prevent differentiation (20-40%). C2C12 cells were cultured in T75 flasks (PAA, Switzerland) in growth medium containing Dulbecco’s Modified Eagle Medium (DMEM) containing 4.5% glucose, 1 mM sodium pyruvate and supplemented with 2 mM L-glutamine and 5 or 20% foetal bovine serum (FBS) (Tab. 3.6). Notably, cell passaging had to be conducted every 2 days for cells grown with 5% FBS and every 1.5 days when grown with 20% FBS. If specified, a modified growth medium was used for on-chip culture that was based on phenol red-free
medium (Tab. 3.7). This medium was chosen to avoid high background signal originating from the phenol red during fluorescence imaging.

HEK-293 cells (ATCC, LGC Standards, France) were kept in culture in T25 culture flasks (PAA, Switzerland) between 20% and 80% confluence. Passaging was done twice per week. Culture medium was DMEM containing 1% glucose, 1 mM sodium pyruvate and was supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 1% penicillin-streptomycin and 10% FBS (Tab. 3.8).

**Tab. 3.6:** Growth medium for C2C12 murine myoblasts.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Supplier</th>
<th>Reference-no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM)</td>
<td>95% or 80%</td>
<td>Life Technologies</td>
<td>41966-029</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS)</td>
<td>5% or 20%</td>
<td>Life Technologies</td>
<td>10270-106</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2 mM</td>
<td>Life Technologies</td>
<td>25030-024</td>
</tr>
</tbody>
</table>

**Tab. 3.7:** Phenol red-free growth medium for C2C12 murine myoblasts for on-chip culture.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Supplier</th>
<th>Reference-no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM)</td>
<td>95% to 80%</td>
<td>Life Technologies</td>
<td>31053-028</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS)</td>
<td>5% to 20%</td>
<td>Life Technologies</td>
<td>10270-106</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2 mM</td>
<td>Life Technologies</td>
<td>25030-024</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>1 mM</td>
<td>Sigma-Aldrich</td>
<td>S8636-100ML</td>
</tr>
</tbody>
</table>

**Tab. 3.8:** Growth medium for HEK-293 cells.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Supplier</th>
<th>Reference-no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM)</td>
<td>90%</td>
<td>Life Technologies</td>
<td>11885-084</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS)</td>
<td>10%</td>
<td>Life Technologies</td>
<td>10270-106</td>
</tr>
<tr>
<td>L-glutamine + penicillin-streptomycin</td>
<td>2 mM + 1%</td>
<td>Life Technologies</td>
<td>10378-016</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
<td>1%</td>
<td>PAA</td>
<td>M11-003</td>
</tr>
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</table>
Chapter 4

An adaptable stage perfusion incubator for the controlled cultivation of C2C12 myoblasts

Here we present a stage perfusion incubation system that allows for the cultivation of mammalian cells within PDMS microfluidic devices for long-term microscopic examination and analysis. The custom-built stage perfusion incubator is adaptable to any x–y microscope stage and is enabled for temperature, gas and humidity control as well as equipped with chip and tubing holder. The applied double-layered microfluidic chip allows the predetermined positioning and concentration of cells while the gas permeable PDMS material facilitates pH control via CO$_2$ levels throughout the chip. We demonstrate the functionality of this system by culturing C2C12 murine myoblasts in buffer free medium within its confines for up to 26 hours. We moreover demonstrated the system's compatibility with various chip configurations, other cells lines (HEK-293 cells) and for longer-term culturing. The cost-efficient system is applicable for any type of PDMS-based cell culture system. Detailed technical drawings and specification to reproduce this perfusion incubation system is provided.

This chapter was published in:
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http://pubs.rsc.org/en/Content/ArticleLanding/2015/AN/C4AN01758G#!divAbstract

Contributions
F. Kurth designed the incubation system and the experimental strategy, designed the microfluidic chip, planned the experiments, conducted all experimental work, evaluated all data, and wrote the manuscript. A.Franco-Obregón planned experiments and wrote the manuscript. C.A. Bärtschi designed and built the incubation system, and proofread the manuscript. P.S. Dittrich planned experiments and wrote the manuscript.
4.1 Introduction

Since the first development of microfluidic platforms for cell culturing and analyses in the mid 90s, technological innovations have greatly improved their utility in basic research and drug discovery (171, 272). Microfluidic platforms are conducive for precise cell manipulation and positioning (175, 176) as well as allow for an unprecedented high level of control over the cellular environment with respect to chemical gradients and biomechanical conditions (259, 273). As much as the elaboration of more complicated designs will lay the foundation for advances in in vitro cell studies, so will the more precise control over basic biological parameters such as temperature, pH and humidity (173).

Several strategies are available to tackle these challenges. The simplest solution is to directly place the entire microfluidic device inside a conventional cell culture incubator maintaining a constant humidified environment at 37 °C and equilibrated with CO₂ (274, 275). The drawback of this strategy, however, is that live cell imaging is significantly hampered. Whereas temperature control is relatively straight forward to achieve and various solutions are available (173), pH regulation commonly relies on the use of buffers with implicit biochemical limitations, such as HEPES (276). Moreover, for the monitoring of certain biological processes, such as transmembrane channel dynamics, the use of particular buffers should be restricted as they may modulate the response (56, 277). Furthermore, HEPES possesses the serious drawback of generating reactive oxygen species when exposed to light (278). To overcome these limitations, custom-made concepts have been developed for the specific control of pH that inherent to this requirement often restricts the device design and needs to be technically adapted for each new task (279, 280). CO₂-independent medium is available for the growing of cells, but is limited in the amount of time that cells can remain viable. A few commercially available systems also provide well-established incubation chambers for microscopes. They provide stable conditions for which to set basic parameters, allow live cell imaging and can occasionally be modified to the user's specific needs. Yet, these systems are expensive, bulky and restricted in use with only one type of microscope setup. More flexible commercially available stage incubation chambers have been developed within the last years that are comparable in performance to the much larger microscope-housing models as well as compatible with standard multi-well plates or cell culture dishes. Only a few of them, however, are amenable for use with common microfluidic devices including their necessity for multiple-port tubing access (281). In addition, a small and microscope-independent incubation system would be required in applications where the microchip requires transportation for use in the field (7). The recent appearance of publications featuring stand-alone stage incubation chambers for microfluidic devices reflect the perceived requirement by the scientific community for such systems (282).

Herein we report on a stage perfusion incubation chamber that can be used in conjunction with common microfluidic devices for the cultivation of mammalian cells (Fig. 4.1). The system is adaptable to any x–y stage of a conventional inverted microscope and regulates temperature and medium pH. It allows long-term observation of cultures on chip and provides gas tight tubing access for media perfusion as well as pressure lines used for the actuation of chip incorporated valves and other features that are based on flexible membranes (154, 199, 283). The pH regulation is achieved by the introduction of a CO₂-containing gas mixture compatible with buffer free medium. The circular shaped chamber is composed of an aluminium alloy for effective heat transfer originating from incorporated resistance heaters for temperature control.
Fig. 4.1: (a) A schematic of the assembled stage perfusion incubation system. (b) An exploded view of the incubation chamber showing all essential parts. (c) Photograph of the open chamber including the embedded chip with tubing connections shown in the middle. Specifically marked are the liquid reservoirs that account for the humidified atmosphere inside the chamber and the gas supply tubing. Tubing access ports that are not to be used will be sealed by blind caps. The outer diameter of the main body is 11.9 cm. Detailed technical drawings (Fig. 4.2 and 4.3) and CAD files (electronic supplementary information, ESI; Analyst webpage) are available.

Closable cutouts in the lid and the bottom of the chamber are amenable for bright field and fluorescence microscopy. Tubing ports are supplied along the sidewall of the chamber, granting access for multiple perfusion or pressure lines. The temperature is regulated by a closed loop control system and gas-tight sealing is achieved using either o-rings or flat gaskets between the lids and the chip. A gas stream is introduced via a high accuracy pressure regulator, supplying a constant CO₂ feed at low flow rates. The diffusion of CO₂ gas into cultivation sites on the chip was assured with the use of the elastomer, PDMS, in chip fabrication. PDMS is frequently used in microfluidic technology for its biocompatibility and gas permeability (153). The material costs for the portable incubation chamber summed to approximately 500 Euro (in 2014).

4.2 Experimental

Working principle of the stage perfusion incubation system

Two feedback regulated resistance heaters (Arcol, HS25 Aluminium Housed Resistor, Switzerland) control the temperature of the incubator; for this purpose a PT100 sensor is adapted into the main body frame.
A defined gas mixture (7% CO₂, 93% synthetic air, Pangas, Switzerland) is applied for the control of the pH of the cell culture medium. The gas flow into the incubator is controlled by a high accuracy single stage diaphragm pressure regulator (Beswick, Greenland, NH) equipped with fluorinated ethylene propylene (FEP) tubing of specified length, accurately regulating the gas supply to a predefined pressure of 25 mbar. The gas mixture is sterile filtered (0.22 mm) prior to entering the incubation chamber through a FEP tubing of 200 mm length and 0.25 mm ID. A final gas mixture flow rate of 4.23 ml min⁻¹ is achieved into the incubator chamber based on the following equation:

\[
\bar{V} = \frac{\pi \Delta p l}{8 \mu}
\]

(equation 4.1)

with \( \bar{V} \) as the volumetric flow rate, \( p \) the gas pressure, \( r \) the tubing ID, \( l \) the tubing length and \( \mu \) the gas viscosity.

To compensate for liquid evaporation from the open chip reservoir as well as to prevent the formation of air bubbles inside the microfluidic device, the incoming gas is first introduced into a reservoir containing 1 ml ddH₂O that also serves to generate a humidified atmosphere inside the chamber. A stable gas flow through the chamber is enabled by opening one tubing port in the sidewall of the main body, allowing the heated air mixture to pass through the chamber while also preventing the diffusion of colder atmospheric air into the system, which would lead to reduction of CO₂ levels.

A thorough description of the stage perfusion incubation system is given in the following as well as detailed technical drawings (Fig. 4.2 and 4.3). Furthermore, all individual components of the incubation system are listed in table 4.1 and separate Inventor CAD files are available for download (ESI).

**Stage perfusion incubation system**

The incubation system is comprised of a circular main body, fitted with a lid and a frame onto which the main body is fastened and that fits onto a x-y microscope stage. The frame is fabricated from polyoxymethylene, whereas the main body and lid are machined from an anodised aluminium alloy. The chip device is centrally situated within a rectangular cutout of the dimensions of a standard 24 x 40 mm glass cover slip and fixed in place by two screw clamps. For microscopy use a rectangular cutout is provided at the bottom of the main body, possessing a lid that can be clamped into place from below when not in use. Gas-tight sealing of the chip is achieved by pressing it against a flat silicon gasket fitted inside the cutout for the glass cover slip by two clamp holders also secured with silicon flat gaskets. In the centre of the incubator lid is a round cut-out window that is closed by a transparent and removable polycarbonate cap, allowing bright field microscopy. This cap and the incubator lid are placed flush against polytetrafluoroethylene (PTFE) o-rings to guarantee gas tightness. Both are fixed by screws and machined washers. To account for fluid and pressure line control of the chip device, pins are provided all around the sidewall of the main body fitting for 1/16” outer diameter (OD) tubing and finger tight nuts including ferrules. Fluid supply was enabled using fluorinated ethylene propylene (FEP) tubing (1/16” OD, 0.25 mm inner diameter (ID); BGB Analytik, Switzerland) and PTFE tubing of 1/16” OD and 0.8 mm ID (PKM S.A., Switzerland) is used for the pressure lines. All tubing is fixed into inlet ports by polyether ether ketone (PEEK) nuts (10-32 threaded).
and ethylene-tetrafluoroethylene (ETFE) ferrules with stainless steel rings (both Ercatech AG, Bern, Switzerland). The pressure line tubing is connected to the chip via custom-built stainless steel connectors. The fluid supply tubing is directly inserted into the chip device. Access pins that are not in use can also be tightly sealed with blind caps (polypropylene, 10-32 tareded).

**Tab. 4.1:** List of parts of the perfusion incubation system. For more details, please refer to the CAD files in the ESI.

<table>
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<th>Number</th>
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<th>Annotation</th>
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<td>chamber main body</td>
<td>aluminium (AL 6036), anodised</td>
</tr>
<tr>
<td>1</td>
<td>chamber lid</td>
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<td>1</td>
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<td>POM</td>
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<td>1</td>
<td>removable chamber lid</td>
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</tr>
<tr>
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<td>chip clamp</td>
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</tr>
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<td>1</td>
<td>silicon flat gasket</td>
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</tr>
<tr>
<td>20</td>
<td>blind cap</td>
<td>PEEK, ≤ 20 (9 applied in presented setup)</td>
</tr>
<tr>
<td>20</td>
<td>access hole nut</td>
<td>ETFE and stainless steel, ≤ 20 (9 applied in presented setup)</td>
</tr>
<tr>
<td>20</td>
<td>access hole ferrule</td>
<td>ETFE and stainless steel, ≤ 20 (9 applied in presented setup)</td>
</tr>
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</tr>
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<td>3</td>
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<td>1</td>
<td>temperature feedback control unit</td>
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<tr>
<td>1</td>
<td>high accuracy diaphragm pressure regulator</td>
<td>Beswick Engineering, Part No. PRDB-3N1-0</td>
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Fig. 4.2: Technical drawing of the main body of the stage perfusion incubation chamber: top views (a, b) and side view (c) with specific labelling.

Fig. 4.3: Technical drawing of the metal lid of the stage perfusion incubation chamber: (a) top view and (b) side view including specific labelling.
Stage perfusion incubation system

**Fig. 4.4:** Image of the setup for temperature validation of the chip device (here shown outside the incubation chamber). An additional hole (1.5 mm diameter) is punched in the middle of a regularly used PDMS chip device to measure the temperature exactly at the spot where the cell cultivation chambers are. A thermocouple is introduced into this hole so that the temperature can be validated under conditions with an open bottom lid. The PDMS block is bonded onto a 24 x 40 mm glass cover slip.

**Incubation system characterisation**

A thermocouple type K (chromel-alumel, 1/16" OD, custom-built) was implemented for the determination of the chamber air and chip temperatures, $T_{\text{air}}$ and $T_{\text{chip}}$, respectively. The thermocouple was introduced into the chamber via a tubing port in the sidewall of the chamber and placed in the centre of the chamber for $T_{\text{air}}$ determination. $T_{\text{chip}}$ was determined by introducing the thermocouple into a 1.5 mm hole punched in the middle of a test chip (Fig. 4.4).

Fluorescent measurements were taken using 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) as a pH indicator at 1 mM in phosphate buffered saline (PBS; pH 7.4, Life Technologies, Switzerland) and cell culture growth medium (GM). The HPTS stock solution was prepared in ddH$_2$O at 10 mM. Measurements were taken at 37 °C in a humidified environment under non-regulated (no CO$_2$ supply, PBS, GM) and regulated conditions (7% CO$_2$, GM). For regulated measurements, the chip device was additionally incubated in a standard cell culture incubator over night in a humidified atmosphere at 37 °C and 7% CO$_2$ prior to the experiments. Images were taken with an EMCCD camera (iXon, Andor Technologies, Ireland) and a 20 x objective. Data readout was analysed using imageJ (284).

**Peripheral systems**

The incubation system setup was mounted onto an inverse microscope (Olympus IX70 and IX71 series) equipped with a mechanical x–y stage and right handle (IX-MVR). Condenser types were either IX-LWUCD or IX-ULWCD. Fluid control was mediated by a high accuracy syringe pump system (Nemesys, Cetoni, Germany) using a gas tight glass syringe (1 ml or 250 ml, Agilent Technologies, Switzerland) connected to FEP tubing (0.25 mm ID) by a PEEK Luer lock system (10–32 threaded female to female Luer and a finger-tight PEEK nut; Ercatech AG, Switzerland). Nitrogen supply for the control layer was regulated by a pressure system from fluigent (Fluigent, France).
Fig. 4.5: Channel system and functional principle of the double-layered microfluidic device. (a) Micrograph of the core channel system filled with dye for visualisation. The inlet branches into 8 cell culture chambers equipped with two main types of cell catching geometries shown magnified in (b). Scale bars: 1 mm (a) and 500 µm (b). For images of captured and attached cells please refer to figure 10. A movie of attaching cells is provided as a supplementary source (ESI).

Chip design

Our design for the culture of C2C12 cells is a multi-layered microfluidic chip device made of poly-(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning), consisting of a fluid layer and an overlying control layer, separated by a thin and flexible PDMS membrane (134, 199). The layers are fabricated separately and subsequently aligned and fixed for final implementation.

The inlet channel of the fluid layer branches stepwise into eight cultivation chambers of 400 µm width and 2 mm length. The channel height is 40 µm. The control layer is comprised of eight separate channels used for physical manipulation of the cells and cell capture. Six lines of horseshoe-shaped pillar structures account for the localised collection and deposition of cells within the fluid channel. Upon pressurisation of the control layer the deformable PDMS membrane intervening between the two layers is depressed into the fluid layer allowing cell collection (Fig. 4.10a). After sufficient time has been allowed for the collected cells to attach to the fluid layer substrate, the control membrane is withdrawn into its original non-deflected state; cells are then free to migrate onto the channel surface (Fig. 4.10b). In this manner cell migration studies can be conducted within the device. Two control channels located at the first fluid channel branch point enable regulated cell supply to either the upper or the lower 4 cultivation chambers (Fig. 4.5). We further analysed the performance of the pressure control layer in relation to the induced pressure. Details are provided below, the results are depicted in figure 4.6.

For the culture of HEK-293 cells a single-layered chip design with a channel height of 100 µm was used. The chip design is similar to the one used for the culture of C2C12 cells except that the longer culture chambers in the middle of the chip were replaced by two shorter culture chambers in series. A micrograph of this design is depicted in figure 4.11.

All details on the wafer and chip device fabrication are provided in the following sections.
Wafer fabrication

The master molds for the double-layered chip device were prepared using 4” silicon wafers and conducted according to Kuhn et al. (134). Briefly, after dehydration of the silicon wafers for 10 min at 200 °C, SU-8 2050 was spin coated at 3250 rpm (fluid layer) and SU-8 2025 at 2100 rpm (control layer; both MicroChem, Newton, MA) onto the wafers to obtain 40 µm and 30 µm thick layers for the fluid and control layers, respectively. After a soft-bake at 95 °C for 6 min the layers were exposed to UV light (365 nm) with an intensity of 160 mJ cm⁻² (SU-8 2050) and 158 mJ cm⁻² (SU-8 2025) through a transparency photomask (Circuitgraphics, UK) on a mask-aligner (MA-6 mask aligner, Karl Süss). Following a post-exposure bake at 95 °C for 6 min, the non-exposed regions were developed in a SU-8 developer (Microchem, Newton, MA) for 5 min (SU-8 2050) and 4.5 min (SU-8 2025). Finally, both wafers were hard baked at 200 °C for 2 h followed by silanisation over night under vacuum with 1H,1H,2H,2H-perfluorodecyl-dimethylchloro-silane (ABCR, Germany).

The fabrication of the 100 µm high channel system was realised with the following adaptations to the protocol: SU-8 2050 was spin coated at 1500 rpm onto the dehydrated wafer followed by a soft bake for 21 min at 95 °C. The layer was then exposed to UV light (365 nm) at 245 mJ/cm² and post-exposure baked for 10 min at 95 °C prior to a 10 min development.

Chip fabrication

For the production of the control layer PDMS oligomer and hardener were mixed at the ratio 5 : 1, degassed for 15 min and poured over the wafer bearing the control layer features. After a second degassing phase of 30 min, the polymer was pre-polymerised at 80 °C for 30 min. Single chips were then diced out of the polymer block and holes for the control layer were punched with a biopsy puncher (1 mm diameter, Miltex).

In parallel to the control layer production, the fluid layer was fabricated based on a soft PDMS mixture (oligomer to hardener ratio of 20 : 1). The pre-polymer was degassed for 55 min and then spin-coated onto the fluid layer wafer at 1400 rpm resulting in a polymer layer height of approx. 60 µm followed by pre-curing at 80 °C for 15 min. In this manner the fluid layer elaborates both the fluid channels and a 20 µm thick flexible polymeric membrane on top. Both layer productions are timed to be finished simultaneously.

After pre-curing of the layers and preparation of the finished control layer blocks, both layers were aligned under a microscope and bonded by complete curing of the polymer mixtures at 80 °C over night. To finish the chip devices, fluid holes are punched using biopsy punchers (1.5 mm diameter, Miltex). For chip assembly, a 24 x 40 mm borosilicate glass cover slip (Menzel-Gläser, Germany) is sonicated for 30 min in ethanol (analytical grade, Fluka, Switzerland). The PDMS chip and the clean glass cover slip are then bonded together using a plasma cleaner (PDC-32 G, Harrick, NY, USA).

For single-layered chips, a 10 : 1 of PDMS oligomer to hardener ratio was poured over the wafer bearing the flow layer features and cured over night at 80 °C. Single chips were diced out of the PDMS slab and holes for fluid connections were punched with a 1.5 mm biopsy puncher (Miltex). Chip bonding and preparation was conducted identically to the multi-layered chip preparation protocol.
Fig. 4.6: Membrane deflection profiles of the two prominent cell capturing geometries as indicated in a and d. The channels were flushed with a fluorescein solution and each trap type was monitored in the range of $p = 0$ to $p = 900$ mbar with $\Delta p = 100$ mbar. (a-c) Profiles of the long geometry type indicate a maximal deflection of about 25 µm at 900 mbar leaving a gap of ~15 µm in between the deflected membrane and the channel bottom for fluid passage. The gap in between the actuated membrane and the channel bottom is essential, as without it, fluid flow would redirect fluid flow (and cells) around the trap and preclude cell entrapment. The lengthened design allows a higher capturing number than the short type. (d-f) Deflection profiles of the short capturing geometry with a maximal deflection of ~23 µm leave a gap of ~17 µm for fluid flow passage. Scale bars (a, d): 100 µm. A movie file showing cell capture is available in the ESI.

**Chip characterisation**

To validate the performance of the pressure control layer used for the cell traps, fluorescent imaging was conducted to visualise the deformation of the different pad geometries under defined nitrogen pressures ($p$). For this purpose a 10 µM fluorescein in ddH$_2$O solution was flushed through the channels at 1 µl/min. Starting from $p = 0$ mbar images were taken at 10 x magnification with an EMCCD camera (iXon, Andor Technologies, Ireland) and $p$ was increased stepwise ($\Delta p = 100$ mbar) up to 900 mbar, which was the maximal stable pressure supply afforded by the Fluigent system at a maximal incoming pressure. Data analysis was done by imageJ (284). Hereby, the intensity in the 0 mbar micrograph ($I_{0 \text{mbar}}$) was taken equal to the maximal height of the fluid layer mold features ($h_{0 \text{mbar}}$). All other measured intensity values ($I_i$) were converted to the corresponding height ($h_i$) using the following formula:

$$h_i = \frac{I_i}{I_{0 \text{mbar}}} h_{0 \text{mbar}}$$

(equation 4.2)

with $i = 100$ to 900 mbar and $\Delta p = 100$ mbar.

The results of the chip characterisation are depicted in figure 4.6.
Fig. 4.7: Fluid supply reservoir designed for easy accessibility and solution exchange. (left): Technical drawing of the custom-built liquid reservoir fabricated from PMMA. Due to the conical shape at the bottom of the reservoir, cell feed to the chip is not negatively affected by cell sedimentation to the bottom of the reservoir potentially enabling long-term cell feed. (right): Photograph of the PDMS chip embedded in the perfusion incubation chamber including the implemented reservoir. Reservoir and channels are visualised by filling with food dye. The tubing for fluid control is inserted into the chip opposite to the reservoir. All tubing accesses connected to the chip with angled metal connectors (4 on each side of the chip) account for nitrogen pressure supply for the pressure layer.

**Chip preparation**

All channels of the chip were filled with DMEM without supplements (Life Technologies, Switzerland) by centrifugation (800 x g, 5 min) immediately after plasma bonding. As the liquid was pulled through the chip during the experiments, the inlet hole was equipped with a custom-built poly(methyl methacrylate) (PMMA) reservoir for easy fluid exchange (Fig. 4.7). The chip was then equilibrated under a humidified atmosphere at 37 °C and 7% CO₂ over night. The following day, the chip was assembled into the perfusion incubation system and the channel surface was coated with fibronectin (FN; Life Technologies, Switzerland) by flushing the channels with a 0.1 mg ml⁻¹ FN in PBS at -0.2 ml min⁻¹ for at least 30 min. The fluid was exchanged to culture medium prior to cell seeding.

**Cell culture**

C2C12 mouse skeletal myoblasts were obtained from the American Type Culture Collection (ATCC; LGC Standards, France). Culturing was conducted in growth medium (GM) containing
Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Switzerland) including 4.5% glucose, 1 mM sodium pyruvate and 2 mM L-glutamine completed with 20% foetal bovine serum (FBS; Life Technologies, Switzerland) in a humidified atmosphere at 37 °C and 7% CO₂. Cells were passaged every 1.5 d, seeded into 75 cm² flasks (TPP, Switzerland) and kept at low confluences (20% to 40%) to prevent differentiation. For on-chip experiments, cells were seeded in 25 cm² flasks (TPP, Switzerland) and grown until 40% confluence was reached. The cells were trypsinised, re-suspended in 5 ml GM and centrifuged at 200 x g for 5 min. Supernatant was discarded, cells were re-suspended in 1 ml GM and subsequently filtered via a 20 µm pore diameter cell filter (CellTrics, Partec, Germany) for cell suspension supply of single cells to the chip.

Human embryonic kidney cells (HEK-293) were obtained from the American Type Culture Collection (ATCC; LGC Standards, France). Cells were grown in DMEM (Life Technologies, Switzerland) containing 1.0% glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% non-essential amino acids (PAA, Austria), 1% penicillin–streptomycin (Life Technologies, Switzerland) and supplemented with 10% FBS (Life Technologies, Switzerland). Cells were passaged twice a week into 25 cm² culture flasks (TPP, Switzerland) prior full confluence was reached. For passaging, cells were trypsinised, centrifuged at 700 x g for 5 min and seeded at an initial confluence of 20% after re-suspension in fresh medium.

On-chip cell culture

For the on-chip cell seeding of C2C12 cells, the cell suspension was added to the chip reservoir and next drawn through the chip by suction. Cells were collected by pressurising the pressure pad lines with 900 mbar N₂ pressure starting from the last pad in flow direction and sequentially pressurising in a forward direction; cells were fed into the chip at a flow rate of ~2.5 ml min⁻¹. After cell capture, the flow was stopped and the N₂ pressure was released from the control layer. The cell suspension in the chip reservoir was exchanged for fresh GM (equilibrated to 37 °C and 7% CO₂) and flushed into the chip at -0.2 ml min⁻¹ for 10 min to sustain the cells.

For perfusion of C2C12 cultures on chip, the open bottom area of the perfusion chamber was closed with an aluminium lid. Fresh growth medium was supplied every 0.5 h at 0.01 ml min⁻¹ for 5 min, regulated by an automated syringe pump script.

For cell counting images of growing cultures were taken every 2 h by a CCD camera (UK-1117, EHD, Germany) via a 10 x objective. The bottom lid of the perfusion chamber was removed for imaging only. Cell number at each time point was normalised to the original seeding density.

For long-term culturing of HEK-293 cells, the cell suspension was added into the reservoir and flushed into the chip at 1.2 µl min⁻¹ until 20 to 30% confluence was reached. Medium was renewed twice a day at 0.333 ml min⁻¹ for 1 h. The flow speed was chosen to keep the induced shear force at a minimal level, i.e. ≤ 0.1 dyn cm⁻², a rate that is considered unproblematic for most perfusion cultures (173).
Cell viability assay

To evaluate the cell viability trypan blue (Sigma, Switzerland) was used to identify dead cells. Cell culture flasks were rinsed once with phosphate buffered saline (PBS; pH 7.4, Life Technologies, Switzerland) and replaced with a trypan blue buffer solution consisting of a trypan blue to PBS ratio of 1:3. For on-chip cultures the reservoir solution was exchanged directly with the same trypan blue buffer solution and flushed through the chip for 5 min at 0.333 ml min$^{-1}$ prior to imaging using an EMCCD camera (iXon Ultra, Andor Technologies, Ireland) at 20 x magnification. Cell counting was done using Image J (284).

Stress assay

The stress assay was conducted as previously described by Crocetti et al. in 25 cm$^2$ culture flasks (285). Briefly, as a positive control for cell viability under steady state culturing conditions HEK-293 cells were stressed with 1 mM hydrogen peroxide (Merck, Germany) overnight on the fourth day of culturing. The following day (day 5) dead cells were stained with trypan blue as described previously.

4.3 Results and discussion

Temperature and pH control

To evaluate the performance of the temperature control system the inner air temperature ($T_{air}$) and the chip temperature ($T_{chip}$) were recorded for a preset temperature ($T_{set}$) using a thermocouple (Fig. 4.8a) and a gas inlet feed of 4.23 ml min$^{-1}$ (the gas is not pre-heated). For $T_{set}$ the feedback loop control guarantees an accuracy of ±0.1 °C for all tested ranges above room temperature (RT). At a preset temperature of $T_{set} = 37$ °C, $T_{air}$ was measured at 36.63 °C ± 0.25 °C, i.e. 1% aberration. During long-term microscopic observation, for which the bottom lid had to remain open, $T_{chip}$ was recorded with 36.5 °C ± 0.1 °C at $T_{set} = 37$ °C, i.e. 1.35% offset, which could be easily compensated for with only minor $T_{set}$ adjustments. When the bottom lid was closed, $T_{chip}$ was coincident with $T_{set}$, due to the minimal heat drain through the sealed chip base. Offsets at lower temperatures for $T_{air}$ and $T_{chip}$ are affected by room temperature. Therefore, in addition to mammalian cultures (37 °C) our system is also amenable to bacterial and yeast cultures (≤ 37 °C).

Next, a defined gas mixture is fed into the chamber to establish a constant pH of the medium inside the open chip reservoir before being fed into the chip. Due to the gas permeability of PDMS, the pH of the medium inside the chip is controlled, allowing on-chip cell cultivation over longer time periods. We verified the constancy of the pH inside the chip with the pH-sensitive dye 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (Fig. 4.8b). Comparing uncontrolled conditions (no CO$_2$ flow) to CO$_2$-equilibrated conditions demonstrates that pH regulation of the cell culture medium within physiological range is clearly possible with this approach.
Fig. 4.8: (a) Temperature validation of $T_{\text{air}}$ (solid circle) and $T_{\text{chip}}$ (open diamond) of the incubation system including linear fits. $T_{\text{set}}$ (black line) indicates a temperature accuracy of 0.1 °C for all preset values above room temperature. At 37 °C $T_{\text{air}}$ shows an offset of 1% from $T_{\text{set}}$ and $T_{\text{chip}}$ shows an offset of 0.5 °C with the bottom lid open after 1.5 minutes. When the bottom lid is closed $T_{\text{chip}}$ resembles $T_{\text{set}}$ for all measured values. All data points are mean values of three independent measurements. (b) pH control inside the perfusion incubation chamber with and without a constant feed of a defined gas mixture (7% CO$_2$, synthetic air). HPTS is used as a fluorescent pH indicator and PBS (pH = 7.4) as a pH reference solution. At uncontrolled conditions (no CO$_2$ feed) growth medium (GM) clearly depicts a more basic pH (pH = 7.98, measured with regular pH meter). Under controlled atmospheric conditions GM is regulated to physiological pH. All data points include data from three independent measurements that have been normalized to the maximal measured value.
**Fig. 4.9:** Passive cell deposition. Cells were seeded in the same cultivation chambers under identical conditions, but without the application of additional trapping tools. A lower flow rate was chosen to allow cell settlement to the channel surface. The passive cell seeding leads to a steep cell density gradient within the chambers, which is unbenevolent for cell types that are strongly affected by the culture confluence. The image was taken 2 h after cell seeding. For a comparison on how cell confluence benefits from the use of the presented catching structures, please refer to figure 10 (a, b) below. Scale bar: 200 µm.

**Double-layered chip performance**

Here we describe a double-layered PDMS chip, whereby an upper control layer descends a pillar for cell trapping (Fig. 4.10a and b and Fig. 4.5). Such horizontally movable cell traps were used to achieve selective cell seeding at predefined positions within the cultivation chambers. The actuation of the cell traps is achieved by stepwise pressurisation of the control layer from 0 mbar up to 900 mbar thereby regulating the distance between the descending pillar and the channel base (Fig. 4.6). In addition, by pressurising the more distal cell trap first, followed in order (distal to proximal) by the other rows of pillars, a homogeneous distribution of seeded cells along the microchannel is achieved (Fig. 4.10a and b). When a straight chamber is utilised without traps, cells are seeded from high density to low density, front to rear, respectively (Fig. 4.9). For instance, individualised C2C12 cells can be successfully trapped with a pressure of minimally 700 mbar applied to the trap. Once the cells have securely adhered to the chamber substrate (approximately 10 minutes) the traps can be released enabling free migration and division without any obstacles that would have originated from permanent cell hurdles, etc.

It should be noted that the presented approach of removable traps could also be employed for reversible capture or sorting purposes for the selection of suspension cells or particles based on size. Additionally, reversible trap motifs have been previously used for the patterning of cells (283) as well as could potentially be applied to the building of multi-layered cell sheets for tissue engineering purposes (224).
Chapter 4

**Fig. 4.10: (a and b)** Schematics (left) and micrographs (right) of newly captured (above) and adhered cells (below). Upon depression of the membrane cells are trapped between the substrate and the descending pillar (a). Upon adhesion to the substrate, the membrane pillar is retracted back to its original position and the cells can freely migrate and divide over the smooth surface without geometric obstacles (b). Image (b) was taken 14 h after cultivation start. Scale bars: 100 µm. Movies of cell capturing and cell migration and division are available in the ESI. **(c)** C2C12 proliferation rates on chip and in a conventional cell culture flask. Example of representative growth curves obtained from 5 on-chip C2C12 cultures (grey scale). Cell growth in a conventional 75 cm² cell culture flask is shown in red (mean values of 4 counts). Growth rates were independently calculated for 24 hour periods. For the on-chip cultures all doubling times are briefer than 24 h, consistent with cell culture results cited in the literature (35, 72). The mean doubling time for the presented on-chip populations was 16.9 h ± 3.0 h (n = 5), correspondent to a specific growth rate of μ = 0.0409 ± 0.007. The presented conventional cell culture doubling time is 13.3 h ± 2.1 h with a specific growth rate of μ = 0.0523 ± 0.008.

**Cell growth**

The mechanobiological factors regulating the proliferation of C2C12 murine myoblasts have been studied (35, 72). C2C12 cell growth rate was taken as a measure for cell viability within our culturing paradigm. The normal doubling time of C2C12 myoblasts is between 12 and 24 hours (μ > 0.029, μ: specific growth rate) depending on culture conditions and the existence of appropriate mechanical cues (35, 72). Repetitive perfusion with medium every 30 min at low flow rates (i.e. 0.02 dyn cm⁻²) was found to provide the optimal mechanical stimulus for cell growth and viability. Growth rates declined with longer time intervals interspersed between media feeds (data not shown).

Fig. 4.10c illustrates C2C12 growth rate in our microfluidic device compared to cell growth rate in a conventional cell culture flask. Representative growth curves obtained from five independent C2C12 cultures grown on-chip are depicted (grey scale) along with their respective calculated values of growth rate. For comparison, an example of the growth rate observed in a conventional tissue culture flask (75 cm²) is shown in red. C2C12 myoblasts cultured on-chip doubled once during the 24 hour observation period.

The mean calculated growth rate was 16.9 h ± 3.0 h (i.e. μ = 0.0409 ± 0.007), only slightly longer than that cited for cells under conventional tissue culturing conditions, i.e. 14 h, yet significantly faster than that for cells grown while mechanically-unloaded, i.e. 30 h (simulated...
microgravity) (35). In general, rapid cell growth rate at the onset of plating was observed, followed by a slowing in growth rate upon the establishment of cell–cell contact, a process known as contact inhibition and associated with the down-regulation of key components of the cellular mechanotransduction apparatus (72, 286). Mechanical stimulation of myoblasts increases their proliferation (287), whereas mechanical unloading stalls their proliferation (35).

A subset of the myoblast on-chip cultures exhibited relatively rapid growth rates during the first few hours of plating likely due to the mechanical stimulation afforded by intermittent fluid flow (feeding) that was not available in the tissue culture flask (cf. Su et al. 2013) (185). An abrupt reduction in proliferation, however, could also signify that myoblasts on-chip reach confluence earlier and withdraw from the cell cycle sooner than the myoblasts maintained in the tissue culture flasks. In support of this interpretation it was apparent that cells on-chip underwent a deceleration of proliferation before myoblasts grown in standard tissue culture plastic. C2C12 cultures could be cultured on-chip for up to 26 h before significant contact inhibition was apparent. Although plating myoblasts at lower density would prolong how long they would maintain proliferative on-chip before undergoing contact inhibition, they would also run the risk of going quiescent due to low cell density. By necessity, robust proliferation must segue into differentiation, otherwise tissue regeneration would be short-circuited. Longer culturing periods allowing for the fusion of myoblasts into differentiated myotubes were not conducted here, but it is anticipated that cell viability and differentiation would not be compromised as long as media exchange was provided for up to one week, a feature that as we have shown is achievable with this system as the incubation chamber provides stable cell culturing conditions as long as media exchange is maintained.

**Long-term culture viability**

To investigate operational stability and versatility with respect to other chip designs and cell strains experiments were conducted with HEK-293 cells in long-term experiments. To this end, HEK-293 cells were seeded in a modified single-layered chip device (Fig. 4.11a) and cultured for 5 days. At the end of five days cells were tested for Trypan blue inclusion to ascertain viability, dead cells staining blue (Fig. 4.11b). As further reference conditions for our stage incubation system, HEK-293 cells were seeded in an identical chip device as well as in a standard tissue culture flask, but maintained in a conventional cell culture incubator for identical durations. As a positive control another batch of cells were induced to undergo oxidative stress (1 mM hydrogen peroxide) prior to the viability check on day 5.

Fig. 4.12a summarises the results of the long-term cell culture experiment. The on-chip cultures grown on stage demonstrated similar viability to that achieved in the tissue culture flask control grown within an incubator (~98% to 99%), whereas the viability of cultures having undergone oxidative stress and maintained with the incubator was significantly decreased (~85%). The viability of cells cultured on-chip, but maintained inside a conventional cell culture incubator was also similar to that from either the flask-incubator or chip-stage condition. Generally, cultures on-chip reached full confluence at around the same time as the cultures in flasks (Fig. 4.12b). This fact is also reflected by the microscopy images depicted in Fig. 4.12c here shown for day 2 and day 5 of the culture time.
**Fig. 4.11:** (a) Chip design used for the long-term culture of HEK-293 cells. Here, the longer culture chambers of the previous design (see Fig. 4.5) are each replaced by two shorter culture chambers placed in series. In this manner the seeding density gradients as shown in figure 4.12 are avoided. The channels and chambers are 100 µm in height and filled with food dye for visualisation. (b) Cell viability determination using Trypan blue. Trypan blue enters necrotic cells and colours dead cells dark. The left image shows a bright field micrograph of HEK-293 cells in regular cell medium, the right image shows the identical HEK-293 cells after staining by Trypan blue. Dead cells are indicated with red arrows. Scale bars: 1 mm (a), 50 µm (b).

**Fig. 4.12:** (a) HEK-293 viability after 5 days in culture for cells grown in conventional culture flasks and on-chip. Cells grown in conventional culture flasks or on-chip and maintained on stage exhibited similar values of viability, 98% and 99%, respectively. Cells having undergone oxidative stress show significantly reduced viability, 85% (*: p < 0.05). (b) Cell densities after 5 days in culture for all conditions demonstrate that cell growth is not limited in our on-stage incubation system. (c) Selected images of the cell cultures for on-chip and tissue flask cultures on day 2 and day 5 of the trials. Cell confluence is similar in all cases for each day.
4.4 Conclusions

Here we present the design of an inexpensive perfusion incubation chamber for the cultivation of mammalian cells under regulated conditions to be combined with standard PDMS-based microfluidic devices. The incubation chamber is adaptable to any x–y stage of conventional inverted microscopes, but can also be used as a stand-alone cell incubation system; the system is portable as long as it is connected to a portable CO₂ gas mixture flask. The presented concept is especially valuable for the observation of biological processes that are sensitive to buffer-containing media.

Acknowledgements

Funding from the European Research Council under the 7th Framework Programme (ERC Starting Grant no. 203429 μLI- PIDS) is gratefully acknowledged. We thank H. Benz for construction of the electronic heating circuit, P. Kuhn for the fabrication of the master moulds and K. Eyer for fruitful discussions.
Chapter 5

Transient receptor potential vanilloid 2-mediated shear-stress responses in C2C12 myoblasts are regulated by serum and extracellular matrix

The developmental sensitivity of skeletal muscle to mechanical forces is unparalleled in other tissues. Calcium entry via reputedly mechanosensitive transient receptor potential (TRP) channel classes has been shown to play an essential role in both the early proliferative stage and subsequent differentiation of skeletal muscle myoblasts, particularly TRP canonical (TRPC) 1 and TRP vanilloid (TRPV) 2. Here we show that C2C12 murine myoblasts respond to fluid flow-induced shear stress with increments in cytosolic calcium that are largely initiated by the mechanosensitive opening of TRPV2 channels. Response to fluid flow was augmented by growth in low extracellular serum concentration (5 vs. 20% fetal bovine serum) by greater than 9-fold and at 18 h in culture, coincident with the greatest TRPV2 channel expression under identical conditions ($P < 0.02$). Fluid flow responses were also enhanced by substrate functionalization with laminin, rather than with fibronectin, agreeing with previous findings that the gating of TRPV2 is facilitated by laminin. Fluid flow-induced calcium increments were blocked by ruthenium red (27%) and SKF-96365 (38%), whereas they were unaltered by 2-aminoethoxydiphenyl borate, further corroborating that TRPV2 channels play a predominant role in fluid flow mechanosensitivity over that of TRPC1 and TRP melastatin (TRPM) 7.

Key Words: mechanotransduction, calcium, laminin, myogenesis, microfluidics

This chapter was published in:


Contributions

F. Kurth designed the experimental strategy, planned the experiments, conducted all experimental work apart from the expression level quantifications, evaluated all data, and wrote the manuscript. A. Franco-Obregón planned the experiments, judged the data, and wrote the manuscript. M. Casarosa retrieved the expression level quantifications. S.K. Küster wrote the Matlab-script for data evaluation and proofread the manuscript. K. Wuertz-Kozak proofread the manuscript. P.S. Dittrich planned experiments and wrote the manuscript.
5.1 Introduction

Mechanical forces are the most unyielding of all sensory modalities impinging on the cell, encompassing both exogenously derived forces such as the constant force of gravity and endogenously derived forces such as pulsatile and shear forces arising from blood flow through sanguine vessels. The translation of mechanical stimuli into biochemical responses is a process known as cellular mechanotransduction and governs the immediate activation of enzymatic cascades as well as long-term cell fate decisions (1, 288, 289). Although multifaceted in mode of activation, cellular mechanotransduction often impinges on calcium (1, 116, 117). Particularly, mechanical force is a key determinant in the development and ultimate physiological function of skeletal muscle (8, 10, 289, 290), which is inherently influenced by calcium (10, 33, 35, 65, 290).

Calcium choreographs myogenic progression commencing with the early expansion of the myogenic stem cell pool (72) and extending into developmental commitment into mature skeletal muscle (291). Myoplasmic calcium levels, in turn, are established by a combination of transmembrane calcium entry (72) and release from intracellular stores, of which the implicated calcium-entry and calcium-release channels change during myogenic maturation (292). Moreover, both extracellular calcium influx and intracellular calcium release are interdependent processes via 2 mechanisms: 1) calcium-induced calcium release (CICR), whereby calcium influx across the membrane instigates calcium release from intracellular stores (38, 40, 41); and 2) store-operated calcium entry, whereby depletion of intracellular calcium reservoirs results in enhanced calcium influx via store-operated channels (39). The recruitment of distinct calcium entry and release channels endows specificity to the selection of diverse calcium-activated enzymes and genes at each stage of myogenesis. Notably, mechanically gated transient receptor potential (TRP) channels of the canonical and vanilloid classes invoke CICR at the early stages of myogenesis (292, 293) and concomitantly function as store-operated channels (39, 70, 72), and alterations in their normal functioning profoundly alter skeletal muscle development (67, 68, 70, 294).

The initial characterization of mechanically gated calcium channels in C2C12 myoblasts was conducted by direct electrophysiological measurements of single-channel activity using the patch clamp technique (36, 37, 295). Recently, macroscopic cellular mechanosensitive responses have also been detected using the whole-cell recording configuration, employing fluid flow to elicit mechanosensitive channel activity at the single-cell level (296, 297). For the measurement of these shear-induced cell mechanosensitive responses, microfluidic platforms are particularly well suited (259) as the fluid flow within the channels is laminar (uniform) and flow velocities and
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Shear stresses can be precisely applied within the micronewton range where mechanically gated channels have been shown to gate (37, 295-297). Microfluidic platforms can also be readily combined with microscopic methods and fluorescence spectroscopy for the readout of cellular responses, thus circumventing the need for direct physical contact between the plasmalemma and recording device. This mode of force transmission thus allows for the examination of integrated responses from small communities of cells undergoing normal interactions with each other as well as with their extracellular environments.

Mechanically mediated calcium responses are often inferred from conventional single-channel measurements as the single-channel currents measured with calcium as the principal charge carrier are in the subpicoampere range (36, 37, 89) and the contribution of calcium release (via CICR) to the global response is by default commonly ignored due to technological limitations. Recent experiments conducted with calcium-sensitive fluorophores in combination with patch clamping have revealed the existence of localized calcium increments that are highly correlated with single-channel current events (87, 88). At the cellular level, calcium responses to fluid flow have been obtained with microfluidic devices, although the instigating source of calcium entry is often not thoroughly explored (cf. (118, 298)). One recent study has ascribed a defined calcium response to TRPM7 channels, yet in response to undefined fluid shear forces as well as to concentration gradients of growth factors (47).

Here we noninvasively quantified TRP channel activation from small communities of murine myoblasts using fluid flow-induced shear stress (Fig. 5.1). We confirmed the involvement of TRPV2 channels in our detected mechanosensitive cell responses using a pharmacological strategy to dissect the relative contribution of several TRP channel classes known to be expressed in C2C12 myoblasts (40, 56, 57, 72) as well as correlated TRPV2 channel expression with culturing conditions conducive to greatest mechanosensitivity to fluid flow. Often overlooked is the role that the extracellular environment, comprised of both diffusible factors and structural components of the extracellular matrix (ECM), plays in the gating of mechanosensitive TRP channels (1). Our platform allowed us to alter the interaction of C2C12 myoblasts with the ECM to discern modulations in cellular mechanotransduction that were previously only suggested using conventional patch clamp (65) or by examining cellular behavior (33, 287). We also demonstrated that extracellular serum regulates C2C12 mechanosensitivity, although in a more unique manner than previously predicted (47, 78, 112, 299). In summary, our results corroborated and extended previous patch clamp findings examining cellular mechanotransduction, yet in a noninvasive platform that allowed cells to behave unobstructed by the recording paradigm. With our platform, we were able to ascribe a predominant role of TRPV2 in the generation of calcium increments in response to fluid flow shear stress.
Fig. 5.1. A) Photograph showing the microfluidic device to culture and mechanically stimulate myoblasts with fluid flow. The channels are filled with blue dye to visualize the 8 culture chambers per chip (also see Supplemental Information, chapter 5.5). Eight pressure control lines (filled with yellow dye) are used to activate the cell capture features during cell supply. B) Schematic depicting cells undergoing mechanical stimulation by fluid flow-induced shear forces. This experimental platform fabricated from poly-(dimethylsiloxane) (PDMS) allows to simultaneously monitor the effects of distinct ECM components as well as different serum concentrations. C) Illustration depicting the mechanisms contributing to mechanically evoked increases in cytosolic calcium concentration, namely calcium entry through the mechanically gated TRP channels stimulating calcium release from the intracellular stores. The sum of both calcium sources is detected by fluorescence microscopy using the calcium-sensitive dye, Fluo-4.

5.2 Materials and methods

Cell culture

C2C12 murine myoblasts were purchased from the American Type Culture Collection (Manassas, VA, USA; LGC Standards, Molsheim, France). Cells were cultured in growth medium (GM) containing DMEM (Life Technologies, Lucerne, Switzerland) including 4.5% glucose, 1 mM sodium pyruvate and 2 mM L-glutamine supplemented with 5% fetal bovine serum (FBS; Life Technologies) in a humidified atmosphere at 37 °C and 7% CO₂. Cells were passed every 2 d and kept at low confluences (10-40%) to prevent differentiation. For seeding on-chip, cells were trypsinized (TrypLE Express, Life Technologies), resuspended in 1 ml GM and passed through a cell filter (20 µm diameter, CellTrics; Partec, Görlitz, Germany) before flushing through the system. Details on cell seeding on-chip are provided in the On-chip cell culturing section as well as in Kurth et al. (260).
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TRP channel expression level quantification

C2C12 were seeded at a concentration of $0.7 \cdot 10^6$ cells / 25 cm$^2$ flask and grown in GM supplemented with either 5, 10, or 20% FBS and harvested for RNA analysis at 6, 18, or 30 h post plating with TRIzol (TRIzol Reagent; Life Technologies) according to the manufacturer’s instructions. RNA concentration and purity were determined by measuring the 260/280 nm absorption ratio (Nanodrop Lite; Thermo Scientific, Switzerland) and stored at -20 °C until reverse transcription. Reverse transcription was conducted by RT-PCR using primers for TRP canonical (TRPC) 1, TRP vanilloid (TRPV) 2, and TRP melastatin (TRPM) 7 (product numbers: Mm00441975_m1, Mm00449223_m1, and Mm00457998_m1; TaqMan Gene Expression Assays, Applied Biosystems, Switzerland) and expression levels were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase.

Chip design and fabrication

The basic chip design and fabrication have been described previously (260). Briefly, the system consists of a 2-layered microfluidic chip device fabricated from poly-(dimethylsiloxane) (Sylgard 184; Dow Corning, Wiesbaden, Germany), whereby a control layer is aligned over a fluid-filled culture layer. Both layers are separated by a thin and flexible poly-(dimethylsiloxane) membrane and the cell culture layer is closed by a glass cover slip (#1; Menzel-Gläser, Braunschweig, Germany) after plasma exposure. Eight parallel culture chambers are equipped with 3 overhead and serially arranged horseshoe-shaped pillars that are deflected downward upon pressurization of the control layer for capturing cells. After the cells are collected at the defined positions, the flexible membrane is withdrawn, allowing the cells to freely attach and migrate within the rectangular incubation channel. The channel design is illustrated in more detail in the Supplemental Information.

Peripheral systems

The microfluidic chip was embedded in a custom-built stage perfusion incubation system accounting for temperature and pH control (260). The incubation chamber was mounted onto an inverse microscope (Olympus IX70 series; Olympus, Hamburg, Germany) and fluid control was conducted by a syringe pump (Nemesys; Cetoni, Korbussen, Germany). A halide arc lamp (X-Cite 120 PCQ; Lumen Dynamics, Mississauga, ON, Canada) was used for fluorescence excitation at 50% intensity. The excitation light was passed through a neutral density filter (ND-12, 12% transmission; Olympus) and an optical filter set (470/40 nm excitation, 495 nm dichroic, 525/50 nm emission; AHF Analysentechnik, Tübingen, Germany). Images were taken with a 60x water immersion objective and a high-sensitivity electron multiplying charged coupled device camera (iXon; Andor Technologies, Belfast, Northern Ireland).

On-chip cell culturing

All used GM for on-chip culturing were based on phenol red free DMEM without any supplements but 4.5% glucose (Life Technologies) completed with 1 mM sodium pyruvate (Sigma, Buchs, Switzerland) and 2 mM L-glutamine (Life Technologies). Serum contents and additional reagents were added as specifically indicated. In preparation for culturing, the
microfluidic chip was filled with phenol red free DMEM by centrifugation (800 g, 5 min) directly after plasma bonding, allowed to equilibrate within a conventional cell culture incubator for at least 4 h and then implemented into the perfusion incubation chamber. The channel surface was subsequently coated with either laminin (LN) or fibronectin (FN) (both Life Technologies) by flushing the channels with a 0.1 mg/ml LN or FN in PBS (Life Technologies) at 0.2 µl/min for 45 min. Subsequently, the fluid inside the microfluidic chip was exchanged to a specified GM at a flow rate of 0.5 µl/min for 30 min. After cell seeding on-chip as previously described (260), GM was supplied every 30 min for 5 min at 0.01 µl/min by an automated syringe pump for the specified time of culturing. All fluid exchange and actuation were performed in reverse pump mode (i.e., fluids were pulled through the chip). A fluid reservoir at the chip inlet served for fluid supply.

**Fluorescent dye loading**

In preparation for calcium registry, the bathing medium was replaced for serum free GM and perfused through the system at 0.5 µl/min for 2 min. C2C12 myoblasts were loaded on chip with 5 µM Fluo-4 acetoxyethyl ester (Fluo-4 AM; Life Technologies) in serum free GM including 0.02% (w/v) pleuronic F-127 (suitable for cell culture; Sigma) for 10 min at 37 °C. Following loading, the medium in the reservoir was exchanged to the specified GM and flushed into the chip at 0.25 µl/min for 4 min, followed by static conditions for 25 min to allow enzymatic cleavage of the acetoxyethyl ester moieties of the dye.

**Culture medium osmolarity and colloid oncotic pressure**

To test for potential cell swelling induced by hypotonicity (75, 76), we measured the osmolarity of cell culture medium containing 5, 10, or 20% FBS using a freezing point osmometer (Osmomat 3000basic; Gonotec, Berlin, Germany). The measured values were 340 ± 2, 339 ± 2, and 336 ± 2 mOsm/kg, respectively. The change in colloid oncotic pressure was estimated to be below 1% based on the change in total medium protein content (change in serum concentration) and hence, neglected (300).

**Fluid shear-stress stimulation**

Based on the laminar flow profile inside the rectangular culture chambers, fluid flow-induced shear stress was assumed to equal the wall shear rate approximated by the following equation (111):

\[
\tau_{WALL} = \frac{6 \cdot \mu \cdot Q}{w \cdot h^2}
\]  

(equation 5.1)

with \(\tau_{WALL}\) as the wall shear rate; \(\mu\), the fluid viscosity; \(Q\), the volumetric flow rate; \(w\), the channel width; and \(h\), the channel height. Fluid viscosity was assumed to be 1.2·10^{-3} mPa according to Chin et al. (229), the channel width was 400 µm and the channel height 40 µm. If not indicated otherwise, mechanical stimulation of the cells was conducted at a flow rate of 0.5 µl/min, corresponding to a fluid flow induced shear stress of 0.94 dyne/cm², which correlates well with previously reported shear stresses evoking calcium entry (118). Cells were flow-stimulated for 10 min on and 10 min off. Time-lapse fluorescent imaging was initiated at fluid
flow onset and continued for the entire 20 min. A 15 min rest period was introduced between the requisite 3 consecutive stimulation runs to allow for adequate recovery of the cells. Shorter recovery periods did not allow recovery of resting calcium levels to at least ~60% of the preceding baseline levels.

**Mechanical dose responsiveness**

Shear rates (flow rates) were tested commencing at $Q = 10$ nl/min, equivalent to $\tau = 0.01$ dyne/cm$^2$ and continuing to $Q = 500$ nl/min, equivalent to $\tau = 0.94$ dyne/cm$^2$. Flow stability measurements were conducted to confirm that low flow rates displayed a stable and accurate flow regime. Single-particle tracking combined with the mean-squared displacement analysis was used to identify the predominant fluid flow characteristics. Details on the analysis and results are available in the Supplemental Information.

**Reagents**

Ruthenium red (RR, technical grade; Sigma) and SKF-96365 [1-(2-[4-methoxyphenyl]-2-[3-(4-methoxyphenyl)propoxy]ethyl)imidazole] (SKF; Sigma) were stored as 10 mM stock solutions in sterile ddH$_2$O and were administered to cells at 40 and 50 $\mu$M, respectively. 2-Aminoethyl diphenylborinate (2-APB; Sigma) was stored as a 100 mM stock solution in methanol (HPLC grade; Sigma) and administered at a working concentration of 100 $\mu$M. All stocks were kept at -20 °C for a maximum of 2 wk (2-APB: 1 wk) before replenishment and were brought to working strength in phenol red free GM. All channel modulators were tested on C2C12 myoblasts grown in the presence of 5% FBS.

**Data analysis**

Bright field images and time-lapsed fluorescence image series of a region of interest were analyzed using Matlab (Matlab R2012a; MathWorks, Natick, MA, USA). All images were taken in 16-bit grayscale format. First, selected cells and representative background regions were marked manually in the bright field image using the Freehand tool. The fluorescence microscopy image series was subsequently background corrected by subtracting the mean intensity value of a background region from the mean intensity value of each selected cell. The entire MATLAB script is listed in the appendix (A.4.1).

Further analysis was conducted using MS Excel (Microsoft, Redmond, WA, USA). Each cell data set was corrected to its starting mean value ($t = 0$). In cases of increased fluorescent background of the initial image, the corresponding data sets were background corrected to the following mean value ($t = 1$). All data sets were normalized to the mean value at 10 min of the first stimulation under the control conditions (cells seeded on a LN functionalized substrate, cultured in GM completed with 5% serum for 18 h prior to mechanical stimulation), if not otherwise specified.

**Changes in free cytosolic calcium increments**

Changes in the detected fluorescent signal intensity largely reflect changes in free cytosolic calcium increments. Nonetheless, although Fluo-4 AM (Life Technologies) exhibits high affinity
for calcium, manganese, zinc, and lead also bind to the dye at lower affinity and hence may contribute to the fluorescent signal. Furthermore, dye leakage into the extracellular space, sequestration within intracellular organelles and bleaching also influence the fluorescent signal (85, 301, 302). Of all these confounding factors, photobleaching is likely the most critical in our experimental paradigm due to our extended measuring intervals. To this end, we conducted a control experiment whereby we tested dye bleaching in solution (Fluo-4 pentapotassium salt, 1 µM) in HBSS (1.26 mM calcium; both Life Technologies) using the identical optical setup as for the cell experiments (data not shown). We did not detect any noticeable Fluo-4 photobleaching in cell free solution and hence did not correct for it in our cell experiments.

Statistical evaluation
Each individual data set contained all single cell data obtained under one particular culture condition and shear force. Each data set was individually tested for normal distribution using a 1-sample Shapiro-Wilk test. Samples were considered normally distributed for $P > 0.05$. A 2-sample test for variance was applied to test the homogeneity of variance for each compared data sets. Homogeneity in variance was accepted if $P > 0.05$. A 2-sample Student’s $t$ test was applied for normally distributed samples with equal variance. If homogeneity had to be discarded, a 2-sample Welch test was addressed instead, and if data sets were not normally distributed but homogeneity of the data sets could be assumed, a Mann-Whitney $U$ test was chosen. Statistical significance in Fig. 5.5E was calculated using the 2-sample Welch test for all cases. All calculations were conducted using OriginPro (version 9.0; OriginLab Corp., Northampton, MA, USA).

Kinetic description
Details on the kinetic evaluation of channel activation and cell recovery are available in the Supplemental Information.

5.3 Results

Characterization of the cellular response and validation of the experimental platform

Certain aspects of mechanotransduction can be attributed to the activity of mechanically gated stretch-activated cation channels (10, 38, 40, 292). Fluid shear stress evokes calcium entry in diverse cell classes as well as has the potential to be applied to cells with good reproducibility (47, 296, 297). To assess fluid shear stress-mediated mechanotransduction in our platform, we loaded C2C12 murine myoblasts with the calcium-sensitive fluorescent dye Fluo-4 AM and monitored their changes in cytosolic calcium in response to dynamic fluid flow. Myoblasts subjected to fluid flow displayed an increase in global intracellular fluorescence emission (Fig. 5.2A). Figure 5.2B shows the mean increase in fluorescence from a small community of cells during and after flow stimulation for 3 consecutive trials where the rise and fall in cell fluorescence closely coincided with the onset and termination of fluid flow (Fig. 5.2B).
Fig. 5.2. Inherent mechanosensitivity of C2C12 myoblasts. A) Bright-field and fluorescent micrographs of C2C12 murine myoblasts before (middle) and after (right) fluid flow at 0.94 dyn/cm², demonstrating mechanically induced increments in cytosolic calcium. Scale bars, 25 µm. B) Mean response time course for cells stimulated on chip to fluid flow at 0.94 dyn/cm² for 3 consecutive exposures. Each exposure consisted of 10 min flow stimulation followed by 10 min of recovery. A 15 min pause intervened between each stimulation trial so that the cytosolic calcium levels could sufficiently decrease prior to the next stimulation. For each trial mean values ± SD (gray areas) are plotted, all normalized to the mean intensity after 10 min flow stimulation of the first trial. Left to right: n (cells) = 30, 24, 20; n (experiments) = 5, 5, 5. C) Box plot representation of fluorescence values for each exposure after 10 min of flow stimulation at 0.94 dyn/cm². Individual cell values are depicted adjacent to each box and experiment and cell numbers are equivalent to the numbers given in (B). D) Initial calcium increment as a function of shear forces within the range of 0.09 to 0.94 dyn/cm², displaying dose responsiveness. All values represent the maximum calcium entry for the first stimulation after 18 h in culture from myoblasts plated on LN-treated substrates for the given shear forces. Left to right: n (cells) = 7, 5, 13, 13, 30; n (experiments) = 1, 1, 2, 2, 5.
Fig. 5.3. Influence of serum concentration, time in culture, and ECM composition over myoblast mechanosensitivity. A) Cellular mechanosensitivity is augmented by low serum concentrations after 18 h in culture. Maximum calcium levels differed significantly in all cases, at least $P < 10^{-6}$. Left to right: $n$ (cells) = 30, 24, 20 (1st), 15, 15, 15 (2nd), 6, 6, 6 (3rd); $n$ (experiments) = 5 (5%), 2 (10%), 1 (20%). Black: first exposure; gray: second exposure; light gray: third exposure to fluid flow. B) Extracellular serum content had a lesser influence over cellular mechanosensitivity at 12 h in culture. Left to right:
on the other hand, flow. Figure used in the culturing of C2C12 myoblasts, calcium entry was nearly ablated in response to fluid increase in mechanically extracellular serum (v/v). Reducing serum concentration stimulated calcium responses of cells cultured for 18 h not commonly used with replica macroscopic mechanotransduction responses that recapitulate many of the same characteristics previously observed in single-channel measurements.

Extraneous factors regulating sensitivity to fluid shear stress

Mechanosensitive channel expression level is developmentally regulated to coincide with the calcium requirements of myogenesis (72). We examined how 2 factors known to modulate myogenic progression, time in culture, and serum concentration, influence fluid shear-stress-induced calcium entry and by inference, the functional expression of mechanically gated calcium-permeable channels. Mechanosensitive responses to fluid flow were greatest at 18 h in culture particularly, yet somewhat unexpectedly, when grown in a reduced serum environment not commonly used with C2C12 myoblasts (35-37, 65, 72). Figure 5.3A shows the fluid flow-stimulated calcium responses of cells cultured for 18 h on-chip in the presence of 5, 10, or 20% extracellular serum (v/v). Reducing serum concentration to 5 from 10% led to a significant increase in mechanically mediated calcium entry. In the presence of 20% FBS, more commonly used in the culturing of C2C12 myoblasts, calcium entry was nearly ablated in response to fluid flow. Figure 5.3B shows the results from this same set of parameters applied to myoblasts at 12 h in culture. As before, 20% serum nearly abolished mechanosensitive calcium entry at 12 h.

The magnitude of the calcium response progressively decreased with repeated exposure to identical flow stimulation, reflecting a process of desensitization or habituation to the mechanical stimulus, a hallmark of mechanically gated calcium entry observed during patch-clamp recording (Fig. 5.2B, left to right) (92, 303). Figure 5.2C depicts the spread of the calcium response for the first, second, and third expositions to fluid flow. The overall magnitude of the calcium responses decreased after each successive exposure to fluid flow, exhibiting the strongest degree of desensitization following the first exposition, consistent with patch clamp measurements (92). Figure 5.2D illustrates that the calcium response of the first exposition was graded with the amplitude of the mechanical stimuli, also in good agreement with channel-mediated mechanotransduction (8, 37, 92). Our noninvasive experimental platform thus allows the observation of community-based macroscopic mechanotransduction responses that recapitulate many of the same characteristics previously observed in single-channel measurements.
Fig. 5.4. TRP channel expression in response to serum content and time in culture. Gene expression of TRPV2 (A), TRPM7 (B), and TRPC1 (C) channels from myoblasts grown in the presence of either low (5%) or high (20%) serum after 6, 18, or 30 h of plating. Channel expression tended to be higher in low serum conditions with significance achieved by TRPV2 at 18 h relative to both 6 and 30 h in culture and for TRPC1 at 18 h relative to 30 h in culture. All values are normalized to their corresponding maximum expression level and calculated from 3 independent PCR samples.

LN stimulates the proliferation (287, 304), differentiation (33, 305), and stretch-activated channel activity of muscle cells (65), likely reflecting its suspected influence over the gating mechanisms of either TRPC1 (36, 37, 51, 65, 66, 306, 307) and/or TRPV2 (68, 69, 293). To elucidate the impact of ECM composition on cellular mechanotransduction, myoblasts were grown on substrates conditioned with either FN or LN and their responses to fluid shear stress quantified. The mechanobiologic responses of myoblasts grown on LN were significantly larger than those for myoblasts grown on FN (Fig. 5.3C), paralleling earlier reports that LN enhances mechanosensitivity, proliferation, and myogenic progression to a greater degree than FN (65, 287, 305). LN significantly enhanced mechanosensitivity in low serum (Fig. 5.3D; cf. Fig. 5.3A), yet gave virtually no change at 20% serum (Fig. 5.3E). Our results thus suggest that low serum and LN are impinging on a common mechanotransduction apparatus and agree with previous reports indicating that LN contributes to an ECM that transmits mechanical force to either TRPC1 (51, 66) or TRPV2 (68-70, 78) via the dystrophin-glycoprotein complex.

TRPV2 channel expression mirrors mechanosensitivity in response to serum content and culture time

Stretch-activated channel expression is highest in myoblasts during log-phase expansion of the myoblast pool (37) and correlates more closely with TRPC1 expression than with TRPM7 expression (72). Given our results showing that LN increases mechanosensitivity (Fig. 5.3) in conjunction with previous studies showing that mechanical stimulation via LN stimulates proliferation (287, 308), we sought to determine which TRP channel class best correlated with fluid flow-induced calcium increments. Figure 5.4 shows the expression patterns of TRPV2, TRPM7, and TRPC1 at 5 and 20% serum and at 6, 18, and 30 h in culture. Low serum increased the expression of both TRPC1 and TRPV2 at 18 h of culturing with low and high significance, respectively. On the other hand, 20% serum had no effect over TRPV2 expression at any time point, but increased TRPC1 after 18 h in culture. In summary, highest TRPV2 expression and mechanosensitivity coincide with respect to the time in culture (18 h; Fig. 5.4A and Fig. 5.3A, B,
respectively) and serum content (5% FBS; Fig. 5.4A and Fig. 5.3A, D, respectively) as well as share sensitivity to LN (Fig 5.3E) (cf. (68-70, 78)).

**TRP channel antagonists RR and SKF mitigate mechanosensitive calcium responses**

We next monitored the effects of described TRP channel blockers over fluid flow-induced calcium increments (Fig. 5.5). RR and SKF have been reported to preferentially block TRPV1-3 channel isoforms (58, 76, 80, 309-311), whereas by some accounts TRPC1 (55) and TRPM7 (312-314) are insensitive to both agents. Accordingly, both RR and SKF blocked fluid flow-mediated calcium entry in myoblasts plated on either LN or FN with high significance (Fig. 5.5E). Importantly, whereas RR (Fig. 5.5B, D) and SKF (Fig. 5.5A, C) attenuated the magnitude of the calcium response, LN (Fig. 5.5A, B) and FN (Fig. 5.5C, D) altered the time course of deactivation independently of antagonist (see also Supplemental Table 5.1), indicating that these ECM proteins act on the mechanical level, whereas the antagonists merely block the communication of the mechanical signal. 2-APB (100 µM), while acting as an antagonist for TRPC1 (82) and TRPM7 (315), has been shown to either activate (80, 310, 316) or have no effect over TRPV2 (58). Although a modest trend toward increased calcium levels was uniquely apparent with 2-APB, it neither significantly altered the fluid flow-induced cytoplasmic calcium increments (Fig. 5.6A) nor their kinetics (Fig. 5.6B) when compared with untreated myoblasts. The sum of our results thus supports a predominant role for TRPV2 in the fluid flow-mediated calcium responses.
Fig. 5.5. RR and SKF attenuate cellular response to fluid flow. A-D) Mean response time course ± SD (gray shaded areas) for LN + SKF (A), LN + RR (B), FN + SKF (C), and FN + RR (D). The kinetic time constants are given in Supplemental Table 5.1 in the Supplemental Information (chapter 5.5). Cell numbers are identical as in (E). All fluid flow stimulation was applied at 0.94 dyne/cm² to myoblast cultures at 18 h in 5% serum to address the state of highest mechanosensitivity (i.e., channel activity). E) Maximal cytosolic calcium increments of the first stimulation trial for cells grown on LN (left) or FN (right) in the presence of RR (40 µM) or SKF (50 µM). In response to treatment with RR, cytosolic...
calcium levels decreased by 27 and 28% for myoblasts plated on LN and FN, respectively. SKF reduced cytosolic calcium levels by 38 and 41% for myoblasts grown on LN and FN, respectively; $P < 0.01$ at least for myoblasts on LN and $P < 0.05$ at least for myoblasts on FN. Values were normalized to their control values for LN and FN each. Left to right: $n$ (cells): LN = 30 (control), 15 (RR), 6 (SKF) and FN = 22 (control), 6 (RR), 13 (SKF); $n$ (experiments): LN = 5 (control), 2 (RR), 1 (SKF) and FN = 3 (control), 1 (RR), 2 (SKF).

Fig. 5.6. 2-APB exerts a modest influence over flow-induced calcium increments. A) C2C12 myoblasts in the presence of 2-APB (100 µM; right) did not show significant differences in mechanically mediated calcium entry compared with untreated cells (control, left). $n$ (untreated cells) = 30, 24, 20 and $n$ (2-APB treated cells) = 15, 13, 13; $n$ (experiments untreated) = 5, 5, 5 and $n$ (experiments with 2-APB) = 2, 2, 2. B) Mean response time course of untreated [solid symbols; $n$ (cells) = 30, $n$ (experiments) = 5] and 2-APB-treated myoblasts [open symbols; $n$ (cells) = 15, $n$ (experiments) = 2] ± SD (gray shaded areas). All cells were cultured for 18 h on LN at low serum content (5%) and underwent flow exposure at 0.94 dyne/cm$^2$.

5.4 Discussion

Calcium plays a pivotal role in muscle development and function (10). Mechanically gated calcium entry during early myogenesis was described over 2 decades ago (36, 37) and has since been narrowed to implicate the involvement of TRP channel classes (41, 72, 292, 307). Functional studies examining the mechanoregulation of TRP channels have largely relied on the patch-clamp technique that is limited to how a small patch of membrane immobilized within the confines of a glass capillary pipette behaves in response to a holding voltage and suction applied through the electrode (317). Expectedly, some skepticism has arisen concerning the relevance of using membrane distention to activate channels within a spherical membrane bleb whose geometry changes with time during recording and is for all practical purposes isolated from the rest of the cell mechanically, structurally, and to a large degree biochemically (97-99, 317). This caveat has been largely circumvented with the application of the whole-cell recording paradigm, yet possessing the disadvantage that the cell’s interior must be dialyzed with contents of the recording pipette to establish electrical continuity, thereby impairing the function of essential intracellular mediators such as calcium (93). The experimental platform described in this report
allows for the examination of calcium-mediated mechanotransduction from small communities of cells without interfering with their endogenous mechanobiological or enzymatic processes while freely allowing cell-to-cell communication during registry.

Diverse TRP channel classes have been implicated in cellular mechanotransduction (56, 82, 318, 319). The TRP channels most commonly ascribed with mechanosensitivity are of the canonical (TRPC), vanilloid (TRPV), and melastatin (TRPM) subfamilies (47, 68, 71, 307, 319-322). Accepted fundamental characteristics of TRP-based mechanotransduction include channel activation and deactivation that temporally coincide with the onset and termination of mechanical stimulation, increased channel open probability graded in magnitude to the mechanical stimulus (37, 92, 303), and time-dependent habituation/desensitization of the response (92, 303). Our mechanical stimulation platform produced intact cellular responses revealing these same 3 basic characteristics: 1) mechanically induced activation and deactivation (Fig. 5.2B); 2) mechanical dose responsiveness (Fig. 5.2E); and 3) time-dependent desensitization (Fig. 5.3), consistent with the interpretation that they arose from a population of TRP channels gated by applied shear force.

Although offering the benefits of minimally invasive measurements, our platform monitors global cellular calcium responses arising from mixed populations of TRP channel classes whose relative contributions change with developmental status (72) as well as prime the release of calcium from intracellular stores (38, 41) that moreover regulates their function (82). Unfortunately, most commercially available and broadly tested TRP channel antagonists are somewhat promiscuous in action and block TRP channels across different classes (40, 56, 57). Nonetheless, some determination of the relative contribution of a TRP channel class in an observed mechanosensitive response can be obtained by evaluating the diverse effects of several TRP modulators with pharmacologic hierarchy in tandem. A predominant role for TRPV2 in mediating cellular mechanotransduction responses to fluid flow shear stress was revealed under our recording paradigm based on antagonism by RR (76, 80, 309-311) and SKF (310) (Fig. 5.5) but not 2-APB (58) (Fig. 5.6).

We observed enhanced sensitivity to fluid flow under growth conditions (low serum) that also augmented TRPV2 expression (Fig. 5.3A, 5.4A). Upon first impression, this result seems to be contradictory to previous findings that transient administration of growth factor components of serum stimulate TRPV2 channel activity (78, 112, 309). Underlying this discrepancy is a preferential up-regulation of TRPV2 channel expression in response to growth under low serum conditions (Fig. 5.4), possibly in an attempt to compensate for low serum stimulation. Alternatively, TRPV2 up-regulation may be the result of altered developmental progression in low serum, whereby the time course of channel developmental expression is changed (cf. (72)) and mirrored by corresponding changes in mechanosensitivity. Future investigation would be required to resolve this issue. Nonetheless, growth in low serum provided us with an opportunity to disproportionately increase the expression of TRPV2 to cross-correlate channel expression with mechanosensitivity without requiring transfection of foreign genetic sequences.

Mechanosensitive TRP channels transmit mechanical signals between the extracellular environment (via the ECM) and cell interior (via the cytoskeleton) and in this manner influence cell behavior. Calcium-mediated mechanical cues guide cell migration (323-325) and are correlated with the expression of TRPM7, TRPC1, and TRPV2 channels (47, 306, 326, 327). Plating C2C12 myoblasts on LN-treated substrates enhances the resting activity of stretch-
activated channels (65) as well as stimulates myoblast migration over that observed with FN (33, 287, 304), indicating that an ECM containing LN preferentially conveys some modalities of mechanical input to a subset of mechanosensitive TRP channels. In agreement, we found that plating myoblasts onto LN-coated fluidic channels enhanced mechanosensitivity compared with myoblasts plated on FN (Fig. 5.3C, E; also see Supplemental Information, chapter 5.5). This interpretation is moreover in concordance with the fact that the dystrophin-glycoprotein complex has been shown to mediate the transfer of mechanical forces to stretch-activated channels by coupling the actin-based cytoskeleton with extracellular LN (320). Evidence now indicates that both TRPV2 (68-70, 78) and TRPC1 (51, 66) are subject to regulation by the dystrophin-glycoprotein complex. Of relevance, the potentiating effect of LN over fluid flow responses was also greatest under conditions that produced the greatest enhancement of TRPV2 expression (Fig. 5.4).

In summary, we showed that cellular mechanotransduction is a dynamic process changing with the developmental status of the cell, trophic influences, or the composition of the ECM. Sensitivity to fluid flow closely coincided with TRPV2 expression, being greatest at 18 h in culture and in response to low serum as well as sharing sensitivity to LN and selected TRP channel antagonists. Thus, the sum of our results supports the interpretation that TRPV2 is the predominant mechanically gated channel contributing to the calcium response to fluid shear stress in myoblasts. Moreover, the presented experimental platform was able to noninvasively extrapolate the contribution of previously described mechanosensitive channel gating properties observed with the patch-clamp technique within a macroscopic and multicellular mechanobiologic response and thus also serves to validate that TRP channels do behave as mechanoreceptors in cells.

Acknowledgements

The authors kindly appreciate fabrication of the master molds by P. Kuhn and thank B. Sebastian for providing the mean squared displacement analysis-Matlab code. Funding from the European Research Council (Starting Grant μLIPIDS, no. 203428) is gratefully acknowledged.
5.5 Supplemental information

Microfluidic chip design

Fig. 5.7: Microfluidic chip design applied for cell culture and mechanical stimulation (260). Different cell capture pads were evaluated resulting in differing cell seeding numbers with type 2 > 3 > 1 > 4. Types 2 and 3 were best suited for our experiments. Insert: C2C12 myoblasts cultured on chip. Scale bar: 50 µm.

Flow stability analysis

For uniform cell stimulation a constant fluid movement is essential. To verify that the induced low flow rates are constant and generate stable shear forces we examined the movement of fluorescent particles within our channels by single-particle tracking (328) and fit their motion to the mean squared displacement (MSD). The particle position evolves from a convolution of diffusion and flow and was calculated as a function of time for a one-dimensional system (329):

$$MSD(\Delta t) = \left[ x(t) - x(t_0) \right]^2,$$

(equation 5.2)

with $x(t)$ as the particle position along the flow axis and $t_0 = 0$. At a constant flow rate in x-axis direction diffusion can be neglected as soon as the fluid movement is significantly faster than the diffusion in axonal flow directory and data of tracked particles match theoretical data of constant flow speeds. To further evaluate constancy of the fluid flow, the MSD can be expressed in a simplified form by the following equation (330):

$$MSD = 2 \cdot q \cdot D \cdot \Delta t^s,$$

(equation 5.3)

in which $q$ is the dimension of the system, $D$ the diffusion coefficient and $s$ denotes the regime of diffusion.
TRPV2 mediates shear-stress responses

<table>
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<tr>
<th>flow rate [µm/s]</th>
<th>s (regime of diffusion) [-]</th>
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<tr>
<td>6.51</td>
<td>1.899 ± 0.025</td>
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<td>13.20</td>
<td>1.968 ± 0.009</td>
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<td>32.55</td>
<td>1.980 ± 0.001</td>
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<td>65.10</td>
<td>1.995 ± 0.001</td>
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<tr>
<td>130.21</td>
<td>1.997 ± 0.001</td>
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\[ D_{\text{theoretical}} = 0.906 \, \mu\text{m}^2/\text{s} \]

Average fitting error based on \( R^2 \): 0.06 ± 0.001%.

**Fig. 5.8:** Mean squared displacement of fluorescent beads validating the fluid flow characteristics.

**Left:** Tracking of particles in fluid flow direction. The lowest applied flow rate shows only minor inaccuracy within the millisecond range, which is due to particle diffusion in flow axis (6.51 µm/s versus \( D_{\text{theoretical}} \)). The mainly applied flow rate of 500 nl/min (65.10 µm/s) is in very good accordance to theoretical data and hence implies a constant fluid flow. **Right:** Regimes of diffusion (s) derived by power law fits to MSD data (equation 5.3). Each value depicts the mean of two separate fits. The deviation of \( s \) at 500 nl/min (65.10 µm/s) to directed movement is very low, i.e. < 0.2%, indicating a constant flow profile without pulsation. Faster flow rates only lead to negligible higher accuracy. Values for \( s \) at lower flow rates are influenced by particle diffusion.

As in our case the particle movement is one-dimensional (\( q = 1 \)), the exponent \( s \) is \( s = 1 \) for free diffusion and \( s = 2 \) for directed motion. For only minor deviations from \( s = 2 \), flow pulsation by the syringe pump or the fluid system can be neglected. The theoretical particle diffusion coefficient was calculated based upon:

\[ D_{\text{theoretical}} = \frac{k_B \cdot T}{3\pi \cdot \eta \cdot d}, \]

(equation 5.4)

with the Boltzmann constant \( k_B = 1.381 \cdot 10^{-23} \text{ JK}^{-1} \), the absolute temperature \( T = 295.15 \text{ K} \), the fluid viscosity of water at the absolute temperature \( \eta = 0.955 \cdot 10^{-3} \text{ Pas} \) and the particle diameter \( d \).

**Flow stability measurements**

Fluid flow rates were tested in reverse pumping mode for 50, 100, 250, 500 and 1000 nl/min, spanning a range beyond the flow rates applied in the cell experiments. Visual tracking of the fluorescent particles (0.01% solid in ddH\(_2\)O working concentration, 0.5 µm diameter, F-8888 Component D, Molecular Probes, Switzerland) was performed with the setup applied for cell experiments (40 x magnification, 10 ms acquisition time, 10 Hz). Calculations were done using MATLAB (MATLAB R2012a, MathWorks, MA, USA). Theoretical fluid movement was defined as a constant movement and calculated based on the cultivation chamber geometries (8 times 400 x 40 µm) and the volumetric flow rate applied. The regime of diffusion (s) was derived from power law fits to MSD data. Figure 5.8 depicts the correlation of experimentally
derived and theoretical particle movements, which show good agreement thereby implying constant fluid flow. The experimental diffusion regimes listed in the adjacent table are further consistent with directed fluid flow movement.

**Kinetic description of channel activation and cell recovery**

Mean data values of each condition were separated in time periods of calcium entry (signal increase) and conditions allowing cellular recovery (signal decay). All data sets were fitted using a single exponential function (equation 5.5) or a linear fit (equation 5.6).

\[
 y = y_0 + A_1 e^{x/\tau_1} \quad \text{(equation 5.5)}
\]

Here \( y_0 \) is the offset, \( A_1 \) the amplitude and \( \tau_1 \) the time constant.

\[
 y = mx + b \quad \text{(equation 5.6)}
\]

Most suitable fits were chosen depending on the overall fitting error. Calcium entry was mostly characterized by a single exponential behavior.

Time constants for few calcium entry data sets had to be approximated by linear fitting as well as all data sets depicting cellular recovery. Hereby the following assumptions were made:

- \( b = 0 \) (for calcium entry)
- \( b = 1 \) (for cellular recovery)

Time constants (\( \tau \)) were then calculated according to equations 5.7 (calcium entry) and 5.8 (cell recovery). The definition of \( \tau \) is listed in equation 5.9.

\[
 \tau = \frac{y(\tau)}{m} \quad \text{(equation 5.7)}
\]

\[
 \tau = \frac{y(\tau) - 1}{m} \quad \text{(equation 5.8)}
\]

\[
 y(\tau) = 1 - \frac{1}{e} \quad \text{(equation 5.9)}
\]

A summary of the determined time constants is listed in table 5.1.
Kinetic description of channel activation and deactivation times

Table 5.1: Summary of the time constants for calcium entry (activation) and cellular recovery (deactivation). Time constants were derived either from a single exponential equation or linear approximation for calcium entry and linear approximation for cellular recovery, respectively. Time constants of channel activation approximated by a linear function are marked with *.

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<th>FIBRONECTIN</th>
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<td></td>
<td>1st</td>
<td>28.92 ± 9.24</td>
<td>17.21 ± 2.07</td>
<td>9.36 ± 1.19</td>
<td>5.85 ± 0.21</td>
<td>13.96 ± 6.78</td>
<td>9.59 ± 0.52</td>
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<td>5 % FBS</td>
<td>2nd</td>
<td>14.72 ± 0.47</td>
<td>27.72 ± 4.83</td>
<td>12.74 ± 4.04</td>
<td>14.42 ± 0.50</td>
<td>16.57 ± 14.57</td>
<td>29.20 ± 2.34</td>
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<td>3rd</td>
<td>11.97 ± 3.56</td>
<td>39.81 ± 5.13</td>
<td>13.42 ± 6.95</td>
<td>18.41 ± 1.55</td>
<td>1.92 ± 0.59</td>
<td>49.51 ± 6.86</td>
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<td>1st</td>
<td>11.70 ± 1.16</td>
<td>9.20 ± 0.18</td>
<td>10.31 ± 1.07</td>
<td>13.06 ± 0.52</td>
<td>17.03 ± 6.52</td>
<td>19.51 ± 0.94</td>
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<td>10 % FBS</td>
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<td>19.70 ± 8.75</td>
<td>20.29 ± 0.78</td>
<td>7.24 ± 1.59</td>
<td>26.20 ± 1.22</td>
<td>8.66 ± 2.70</td>
<td>24.43 ± 0.93</td>
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<td>3rd</td>
<td>11.43 ± 1.92</td>
<td>27.93 ± 1.24</td>
<td>6.62 ± 2.30</td>
<td>26.30 ± 2.80</td>
<td>5.33 ± 2.41</td>
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<td>20 % FBS</td>
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<td>5.16 ± 1.22</td>
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<td>109.81 ± 14.59</td>
<td>8.37 ± 3.70</td>
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<td>3rd</td>
<td>7.52 ± 1.76</td>
<td>162.06 ± 17.72</td>
<td>3.25 ± 8.67</td>
<td>424.03 ± 129.96</td>
<td>3.71 ± 1.15</td>
<td>218.98 ± 43.43</td>
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<td>5 % FBS</td>
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<td>13.88 ± 4.76</td>
<td>18.44 ± 2.82</td>
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<td>13.58 ± 0.76</td>
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<td>23.95 ± 17.93</td>
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<td>15.08 ± 0.49</td>
<td>25.09 ± 3.85</td>
<td>13.87 ± 0.43</td>
<td>18.13 ± 1.36</td>
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<td>5 % FBS</td>
<td>+ SKF</td>
<td>4.62 ± 0.32</td>
<td>30.84 ± 3.15</td>
<td>6.00 ± 1.03</td>
<td>10.02 ± 0.38</td>
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<td>2nd</td>
<td>4.25 ± 1.13</td>
<td>68.38 ± 7.94</td>
<td>2.46 ± 0.58</td>
<td>43.18 ± 5.27</td>
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<td>3rd</td>
<td>2.60 ± 0.41</td>
<td>203.25 ± 86.76</td>
<td>13.65 ± 0.81</td>
<td>7.46 ± 0.39</td>
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<td>5 % FBS</td>
<td>+ 2-APB</td>
<td>-</td>
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<td>5.55 ± 0.07</td>
<td>6.59 ± 0.22</td>
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<td>4.64 ± 0.42</td>
<td>15.25 ± 0.42</td>
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<td>28.90 ± 2.08</td>
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Chapter 6

A world-to-chip interface: microfluidic analysis of in vivo samples

In recent years, numerous novel cell analytical microfluidic platforms have been introduced to unravel cellular processes on the single-cell level and in cell cultures. However, many complex biological processes cannot be studied in these “artificial” systems, but require the natural environment of the cell. This chapter presents a novel strategy to close the gap between in vivo models and on-chip cellular analysis. A world-to-chip interface was developed, with which cells dissected out of in vivo tissue samples can be transferred into and analyzed on a microfluidic platform.
6.1 Introduction to bone mechanobiology

In recent years, numerous novel cell analytical microfluidic platforms have been introduced to unravel cellular processes on the single-cell level in cell culture systems (175, 176, 199, 331). However, many complex biological processes cannot be studied in these “artificial” systems, but require the native environment of the cell. Cell analytical platforms in microfluidics though commonly rely on the use of cell suspensions and cell manipulation in artificial environments (171, 332, 333). Although latest technological advances are able to provide more natural cellular environments in vitro (222, 259), current culture systems cannot equivalently replicate the complex interactions among numerous cellular cues, such as biochemical, mechanical, or electrical signals. The adaptation of bone to mechanical loads in one of these complex processes that cannot be captured in vitro. Analyses of bone adaptation in in vivo models offer valuable insight into the interplay between the external forces and intracellular biochemical responses (13, 334). This way, alterations in protein and gene expression levels can be linked to dynamic changes in mechanical load. As microfluidics offer great potential in biochemical analyses in terms of sensitivity, specificity, sample multiplexing and automation that outmatch standard bench-top approaches, a strategy was developed to combine the benefits of in vivo models and microfluidic technology. The basis for the world-to-chip interface was the application of laser capture microdissection (LCM), by which cells of interest from bone cryosections can be dissected (Fig. 6.1) and transferred into an isolated compartment (vial or well) for subsequent analysis.

The work described in this chapter was realized in cooperation with the Biomechanics group (Prof. Ralph Müller, ETH Zürich). Methods for mechanical in vivo stimulation, tissue harvesting and LCM were established by the Biomechanics group (13, 14, 334-337) and adapted by Andreas J. Trüssel.

Fig. 6.1: (a) Micrograph of a bone cryosection. (b) Fluorescence micrograph of the same cryosection as in (a). Cell nuclei were stained with Hoechst dye for visualization (bright dots). (c) Cryosection after LCM bearing circular holes from which tissue samples were cut out. Scale bars: 200 µm.
6.2 Bridging the gap: towards microfluidic single cell analysis of in vivo stimulated cells

We present a novel strategy to enable cellular analysis of in vivo models in microfluidic devices. The strategy combines microdissection of tissue samples and microfluidics for cell capturing and analysis. The technique is optimized for cell capture and analysis of bone, so spatial information can be linked to important information concerning individual cells’ microenvironments, such as strain energy density. In contrast to state-of-the-art microfluidic devices for cell analysis, which are mostly limited to the use of cell suspensions, the presented study opens up the possibility for cell-directed analysis of in vivo models.

Keywords

Microfluidics, Laser Capture Microdissection, Mechanobiology, Bone Adaptation, Osteocytes

This chapter was published in:

Contributions

F. Kurth designed the experimental strategy, planned and conducted experiments, evaluated data, and wrote the manuscript. R.E. Wilson conducted experiments and proofread the manuscript. A.J. Trüssel designed the experimental strategy, conducted experiments and proofread the manuscript. D.J. Webster and R. Müller designed the experimental strategy and proofread the manuscript. P.S. Dittrich designed the experimental strategy and wrote the manuscript.
Chapter 6

6.2 Conceptual schematics. (a) A bone sample (mouse caudal vertebra) is taken from a mechanically stimulated in vivo model. (b) Single osteocytes are dissected out of the bone cryosections by a focused laser beam and catapulted into a microfluidic device placed above the sample. (c) On-chip single-cell analysis provides information on the genome or proteome level for every single cell. (d) Local information of the cells is preserved allowing reconstruction of the sample.

6.2.1 Introduction

Cell analytical platforms are commonly based on the analysis of cell culture in artificial environments (171, 332, 333). Although these approaches mimic natural cellular environments in vitro (259), it is not possible to recreate all of the complex structural and biochemical interactions that occur in vivo. The complex mechanical interaction between cells and their extracellular environment is especially important in trabecular bone adaptation processes, in which dynamic mechanical loading directs bone formation and resorption responses at the micrometer scale towards an optimal balance between strength and minimal weight. In vivo model analyses of bone adaptation frequently use methods such as micro-computed tomography (µCT), multiscale in-silico modeling, and protein and gene expression analysis by immunohistochemistry (IHC) and bench-top PCR approaches, respectively (13). Despite numerous outcomes in mechanical systems biology, these methods still lack in quantification (IHC) or specificity (PCR) at the single cell level. Here, we report on an approach aiming to bridge the gap between standard in vivo model analyses and the numerous capabilities microfluidics technologies are able to offer.

6.2.2 Concept

The complete strategy is illustrated in figure 6.2. A vertebral sample of an in vivo mouse model is harvested and prepared for microdissection. Single osteocytes are cut out from the sample section and catapulted into the microfluidic chip by means of laser capture microdissection (LCM). The microfluidic device is fixed above the sample section with open channel structures facing the sample. After cell transfer into the capture chambers, the chip device is removed, and the channel system is closed. Using standard microfluidics for reagent supply, cellular DNA/RNA can be visualized for the characterization of the samples in terms of cell number and integrity.

As each cell sample is captured in a separate microfluidic chamber, subsequent cellular analyses can be linked to individual osteocyte locations within the in vivo model. Thus, retrieved cellular data allows the reconstruction of the sample, e.g. by intrinsic information on gene expression profiles.
Fig. 6.3: Scheme of the microchip. Exemplarily, three wells are shown. In total, the device contains 12 wells. (a) Side view: the microfluidic chip is positioned above the histology section. Using laser capture microdissection (LCM), single osteocytes or osteocyte clusters are microdissected and catapulted into 100 µm-deep wells of the chip. (b) The wells are connected by microfluidic channels to deliver staining compounds. Scale bar: 500 µm.

The transfer of cells from the tissue section to the chip device is visualized in figure 6.3. The cross-sectional view in figure 6.3a depicts the dissection and catapulting step. Individual cells are optically identified and can be chosen according to µCT derived strain energy density maps. Osteocytes of interest are cut out using a high-intensity, focused laser beam and subsequently catapulted by defocused laser pulses into cell capture chambers of the microchip positioned above. The cell capture chambers are connected by microfluidic channels for fluid supply (Fig. 6.3b), forming an array of 12 adjacent capture chambers for parallel analysis.

6.2.3 Experimental

The master mold for the chip device was prepared using a 4” silicon wafer. The fluidic mold was fabricated according to (212) apart from the following: first, a positive photoresist (AZ-9260, AZ Electronic Materials) was used to generate 12 µm high round interconnection channels. In a second step the 100 µm high cell capture chambers were fabricated applying a negative photoresist (SU-8 2050, MicroChem Corp.). The polymeric chip was made using standard soft lithography methods applying poly(dimethylsiloxane) (Dow Corning) in a 1:10 curing agent to pre-polymer ratio.

The 6th caudal vertebrae of C57BL/6 mice were harvested, snap frozen, and cryosectioned (12 µm thickness) according to (334). LMD tape (Kawamoto) was used to preserve section morphology and facilitate microdissection. In order to identify osteocytes prior to microdissection and to verify the cell tracking potential of this strategy, cryosections were stained with Hoechst stain (10 µg/ml) to mark the nuclei of embedded osteocytes. A laser capture microdissection instrument (Palm MicroBeam, Zeiss) was used to cut out single or multiple osteocytes and catapult these cells into open microchambers. The PDMS chip was attached to a metal holder above the histology section, and a microchamber was aligned just above the osteocyte of interest prior to microdissection.

After cell dissection, the microchambers were closed by a glass cover slip deploying a custom designed clamp. The chip was connected to tubing and a custom-made pressurization system, allowing for the subsequent supply of reagents and staining fluids. Propidium iodide solution
(1 ng/ml) was flushed into the channels to identify the osteocytes and characterize their integrity within the transferred sample. Fluorescent imaging was performed with an Olympus microscope (IX70) and an EMCCD camera (iXon, Andor Technologies).

### 6.2.4 Results and discussion

The performance of the microdissection process was characterized. First, the scattering of cells upon vertical catapulting onto the microdevice was determined as illustrated in figure 6.4a. The histograms in figures 6.4b and c depict the x and y-axis diversions of 142 samples after catapulting, respectively. Based on these findings, we designed the target microchambers with a diameter of 600 µm. Next, the transfer efficiency of cells from the sample into the microfluidic chambers, i.e. dissection and catapulting, were determined to 48%. In 95% of these cases, we spotted intact, not ruptured cell clusters in the chambers. About 75% of single dissected cell samples in the chambers contained complete transferred cells.

Figure 6.5 illustrates the sample dissection and characterization procedure. Cell clusters (e.g. Fig. 6.5a, 7-10) and single cells (e.g. Fig. 6.5a, 1-6) from distinct bone regions were collected out of the histology section. Negative samples were included to characterize the background signal of the supporting tape (e.g. Fig. 6.5a, 11-12). Figure 6.5b represents a selection of processed on-chip samples verifying intact DNA/RNA information for cell samples after the LCM procedure (Fig. 6.5b, No.9 and No.5) and low background signal intensity (Fig. 6.5b, No.11).

### 6.2.5 Conclusions and outlook

Our results demonstrate that we successfully combined LCM and microfluidic technologies to bridge the gap between *in vivo* models and cell analytical microfluidic platforms. This method can be employed for other tissues, cell cultures, and biofilms. In future studies, we plan to investigate mechanically induced gene expression using qPCR in the microfluidic device to yield spatio-temporal information at single cell resolution.

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**Fig. 6.4:** Characterization of the LCM performance. Cells that are catapulted from the sample reach the wells at different positions in the x-y plane (a). The histograms in (b) and (c) are representative data from 142 dissection and catapulting processes. The marked range in (b) and (c) depicts the diameter of the chip wells, into which the samples are catapulted.
Fig. 6.5: Microdissection, chip filling, and cell tracking: a C57BL/6 mouse caudal vertebra (a, insert) is cryosectioned (12 µm thickness). (a) Micrograph of a bone section after LCM. Marked areas were catapulted into separate chambers of the chip; non-marked holes depict unsuccessful LCM attempts. (b) Micrographs and fluorescent images of selected single (no.9) and multiple (no.5) cells. No.11 depicts a negative control of the tape substrate, showing no significant DNA/RNA fluorescent signal. Scale bars: 500 µm (a), 50 µm (b).

Acknowledgements

We thank G. Kuhn for animal work and the supply of bone samples and B. Sebastian, P.E. Verboket and P. Kuhn for fabrication of silicon wafers. Funding from SystemsX.ch is gratefully acknowledged.
6.3 Toward on-chip RT-qPCR of osteocytes from in vivo bone samples

This chapter describes the development of the world-to-chip interface presented in chapter 6.2 towards a RT-qPCR microfluidic device. It comprises modifications of the chip design and fabrication as well as development and evaluation of microfluidic RT-qPCR assay protocols. Besides cell samples dissected out of bone sections, total RNA probes were employed to validate the amplification cycle efficiencies. Particular focus was laid on the mode of signal detection in combination with the chip design. Furthermore, the choice of detergents and surface coatings to prevent sample and probe loss by absorption to the PDMS surfaces was investigated in detail. All data retrieved from on-chip experiments were additionally set into correlation with bench-top acquired results.

Keywords

World-to-Chip Interface, In Vivo Stimulated Osteocytes, Integrated Microfluidics, Laser Capture Microdissection, RT-qPCR

Contributions

F. Kurth designed the experimental strategy, planned and conducted experiments, evaluated data, and wrote the chapter. A.J. Trüssel designed the experimental strategy, developed and tested PCR assays, and provided the custom-built hot plates. R.E. Wilson supported the development of PCR assays and surfactant testing as well as generated experimental results.
6.3.1 Introduction

Embedded within the calcified bone matrix osteocytes sense mechanical stimuli and regulate bone development by orchestrating osteoblasts and osteoclasts to either generate or resorb bone (11, 338-340). Upon dynamic mechanical loading, bone matrix is formed in areas of high mechanical strain and resorbed in areas of low physical load. The mechanical forces are hereby transduced into biochemical responses within the osteocytes, which vary specific protein expression patterns that induce the release of particular signaling molecules into the bone matrix (338, 339). These biochemical signals in turn instigate or attenuate the activity of osteoblasts and osteoclasts, respectively. For instance, the proteins sclerostin and DKK1 are known to govern osteoblast activity whereas RANKL and OPG function as osteoclast regulators (339). To gather further insight into the complex interplay of dynamic loading and developmental consequence, particular protein expression levels in osteocytes are of intrinsic relevance to better understand the mechanically mediated signal transduction pathways in bone development. As the mechanical load impinging onto the osteocytes inherently depends on their respective microenvironment within the bone matrix, which generates a vast variety of strain density areas, the analysis of single osteocytes in respect to their local origin is essential (13, 14). First studies on the mechanical systems biology of bone cells provided valuable results for small populations of osteocytes (~10) of the same local origin for housekeeping genes (ß-actin) (334). However, due to the relatively high starting mRNA concentration for the bench-top approach, these studies were still limited in the number of targeted genes of interest on the single cell level (13).

To access the mechanically induced protein expression levels, the world-to-chip interface described in chapter 6.2 was expanded with reverse transcription and quantitative PCR (RT-qPCR) functionality. As during bone development the expression levels of multiple proteins in osteocytes intercalate to diverse magnitudes, the microfluidic chip was designed in a way that multiple genes could be targeted for each sample during qPCR. Microfluidic RT-qPCR of in vitro cultures has been reported down to the single cell level (212, 213, 341), whereas RT-qPCR from fragile cells dissected out of tissue sections harvested from in vivo models has so far only been reported in the bench-top approach (334, 342-344). The development of a microfluidic RT-qPCR protocol thus implied adaptations of the relevant bench-top approaches to meet the requirements for on-chip analysis. Besides these essential modifications the development of the microfluidic device necessitated a multi-layered chip design offering liquid control using pneumatic valves providing consecutive and isolated sample preparation and handling. Though multiple protocols for the fabrication of such a device exist (212, 213, 341), none of them presents a solution for sample collection prior to full chip assembly, which is a prerequisite for our strategy. Consequently, we established a fabrication protocol that complies with this particular demand (chapter 3.5.3).
For the development of a RT-qPCR microfluidic chip device for the analysis of osteocytes from bone tissue, the comparison of on-chip results to established bench-top approaches is crucial. Thus bench-top protocols were included to characterize on-chip PCR performance. To simplify the PCR performance characterization, protocols for the analysis of total RNA samples were additionally established for both bench-top and on-chip preparations. For microfluidic device planning, fabrication and operation please refer to the general methods section (chapter 3).

**Bench-top protocol for RT-qPCR of osteocytes and total RNA samples**

Bench-top reverse transcription (RT), cDNA pre-amplification and qPCR were conducted according to the manufacturer’s instructions (TaqMan, Applied Biosystems). Briefly, 10 µl RT master mix (Tab. 6.1) was pipetted into a cap of an Eppendorf tube, which was placed above the sample section so that the targeted cells could be captured directly in solution using laser capture microdissection (LCM; Palm MicroBeam, Zeiss). The samples were spun down and directly transferred to 50 °C for RT in a PCR cycler (Mastercycler personal, vaudaux-ependorf). All samples remained at 50 °C until pre-amplification. Each cryosection was handled for 30 minutes only to prevent significant RNA degradation. For the analysis of RNA samples *Hprt* total RNA (hypoxanthine-guanine phosphoribosyltransferase; Mm00446968_ml, 20x TaqMan Gene Expression Assays, Thermo Fischer Scientific) probe solutions (Tab. 6.2) were directly mixed with the RT master mix at a concentration of 5 µg/ml RNA (corresponding to 10,000 cells, total RNA amount: 100 ng). The RT and pre-amplification cycling program consisted of the reverse transcription (15 minutes at 50 °C), the Taq-enzyme activation (2 minutes at 95 °C) and 15 pre-amplification cycles (15 seconds at 90 °C, 4 minutes at 60 °C). The resulting cDNA solution was then diluted in Tris-EDTA (pH 8.0) buffer at a ratio of 1:3 and stored at -20 °C until qPCR. For the qPCR, 8 µl of freshly prepared PCR master mix (Tab. 6.3) were completed with 2 µl cDNA in a well of a 96 well plate. The PCR thermo cycler (C1000 Touch Thermal Cycler, Bio Rad) program consisted of initial steps at 50 °C for 2 minutes and 10 minutes at 95 °C, followed by 40 amplification cycles (15 seconds at 95 °C and 1 minute at 60 °C).

All solutions were prepared without surfactants unless noted otherwise. In case surfactants were included the final surfactant concentrations were as indicated in the on-chip protocols (next section).

**Tab. 6.1**: Composition of 10 µl RT master mix solution for the bench-top analysis of osteocytes from bone samples and total RNA probes. The solution has to be kept on ice.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-EDTA (with surfactant)</td>
<td>2.2</td>
</tr>
<tr>
<td>0.2x primer/probe mix (Tab. 6.2)</td>
<td>2.5</td>
</tr>
<tr>
<td>CellsDirect 2x reaction mix</td>
<td>5.0</td>
</tr>
<tr>
<td>SuperScript™ III RT/Platinum® Taq mix</td>
<td>0.2</td>
</tr>
<tr>
<td>Ambion SUPERase-inhibitor</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Tab. 6.2: Composition of the 0.2x primer/probe mix solution including specific primers (and total RNA). Volumes are exemplarily listed for one gene of interest and yield 40 reaction aliquots. The solution has to be kept on ice.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-EDTA (with surfactant)</td>
<td>99 (100 minus number of genes of interest)</td>
</tr>
<tr>
<td>Gene Primer/Probe (20x)</td>
<td>1.0 (1 µl for each gene of interest)</td>
</tr>
</tbody>
</table>

Tab. 6.3: Composition of the PCR master mix solution for the bench-top analysis of derived cDNA. The listed total volume is required for each gene of interest. The solution has to be kept on ice.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal PCR Master Mix (2x)</td>
<td>5.0</td>
</tr>
<tr>
<td>Tris-EDTA (with surfactant)</td>
<td>2.5</td>
</tr>
<tr>
<td>Primers and Probe (20x)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

On-chip protocol for RT-qPCR of osteocytes and total RNA samples

After bonding and clamping of the chip device, the control layer was connected to tubing (pre-filled with millipore ddH₂O) and the pressure of the control layer was set to 2.5 bar. All tubing to be used for the supply of reagents to the fluid layer was first cleaned with RNaseZAP® (Applied Biosystems, Switzerland), followed by flushing with millipore ddH₂O and drying with nitrogen. The following experimental description is also illustrated in figure 6.6. RT master mix (Tab. 6.4 for the analysis of bone samples and total RNA probes) was flushed through the main feed channel into the sample chambers at 1.0 bar by opening the relevant valve lines. Once the chambers were filled all valves were closed, the inlet pressure was stopped, and the chip was placed on a custom-built copper hot plate at 51 °C for 15 minutes to initiate the reverse transcription. Although the conventional temperature for the RT is supposed to be 50 °C, we used a slightly higher temperature to account for heat loss due to the experimental setting, which we determined separately using an infrared surface thermometer (TFI 220, Ebro, Switzerland). Pre-amplification of the cDNA was performed in 15 cycles of 25 seconds at 95 °C and 4 minutes at 62 °C by manually placing the chip device onto two separate copper hot plates.

Prior to the addition of the PCR buffer (Tab. 6.5), the feed channel was flushed once with Tris-EDTA buffer including 0.5 mg/ml BSA or 0.1% Tween 20. The PCR buffer was added to the already present mixture by opening the respective valves and transferring both volumes in the second chamber for mixing, which took about 1 hour. All valves were closed as soon as the final total assay volume was reached, i.e. the mixing chamber was filled completely. Once the content was mixed thoroughly, it was split into aliquots by transferring the total volume down the sample line. All valves were closed after all aliquots were accomplished in the PCR chambers.
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Fig. 6.6: Micrograph of one out of 12 sample processing lines for sample capture/introduction, preparation and RT-qPCR. The design allows the targeting of up to 8 genes per sample. Samples are either catapulted or flushed into the sample chamber where RT and pre-amplification is performed. The resulting cDNA solution is mixed with PCR buffer in the mixing chamber and after distribution into the PCR chambers combined with the assay mix (for details refer to text). All valves are shown in red: (1) supply valve, (2) mixing valve, (3) assay feed valve, (4) distribution valve, (5) distribution and assay combination valve. The CAD design of the whole channel system is available in the appendix (Fig. A.3.4).

The assay feed channels were first flushed once with Tris-EDTA including 0.5 mg/ml BSA or 0.1% Tween 20 and next blown dry with nitrogen. Afterwards the assay mix (here including the primers for Hp, Tab. 6.6) was introduced into the reaction chambers adjacent to the aliquot chambers. Next, the valves were actuated so that the solutions inside the assay chambers and the PCR chambers could mix with each other for about 2 hours by diffusion at room temperature. To initiate the PCR the chip was first heated to 95 °C for 10 minutes and subsequently 40 cycles of 25 seconds at 95 °C and 1 minute at 62 °C were conducted. To account for heat loss during imaging on a confocal microscope (LSM510, Zeiss, Germany), the chip device was placed on the 95 °C hot plate for 35 seconds after imaging instead of 25 seconds. Images were taken every 2 or 3 cycles at 10x magnification, which took about 2 minutes per time point.

Tab. 6.4: Composition of 10 µl RT master mix for the on-chip analysis of osteocytes and total RNA probes. The solution has to be kept on ice.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume [µl] osteocytes</th>
<th>Volume [µl] total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-EDTA with surfactant</td>
<td>2.2</td>
<td>1.61</td>
</tr>
<tr>
<td>0.2x primer/probe mix (Tab. 6.2)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>CellsDirect 2x reaction mix</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>SuperScript™ III RT</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Ambion SUPERase-inhibitor</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>5 µg/ml RNA</td>
<td>-</td>
<td>0.59</td>
</tr>
</tbody>
</table>
Tab. 6.5: Composition of the PCR buffer solution for the on-chip analysis of derived cDNA. The listed total volume is required for one on-chip analysis. The solution has to be kept on ice.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Universal PCR Master Mix (2x)</td>
<td>5.76</td>
</tr>
<tr>
<td>Tris-EDTA with surfactant</td>
<td>3.24</td>
</tr>
</tbody>
</table>

Tab. 6.6: Composition of the assay mix for qPCR addressing the gene *Hprt*. The listed volume is sufficient for the filling of one assay line. The solution has to be kept on ice.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-EDTA with surfactant</td>
<td>0.75</td>
</tr>
<tr>
<td>Primers and Probe (<em>Hprt</em>) (20x)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

The final concentration of Tween 20 was 0.1% and 0.5 mg/ml for BSA in all solutions. Polyvinylpyrrolidone (PVP) was added at a final concentration of 2% for microfluidic use and PLL-g-PEG (poly(L-lysine)-g-poly(ethylene glycol)) surface functionalization of the channel walls was performed for 30 minutes directly after plasma activation. All surfactants were added to the Tris-EDTA buffer listed in tables 6.4 – 6.6 when noted. Notably, a wet tissue surrounded the chip device during all temperature cycling procedures to minimize solution loss due to evaporation through the PDMS material at the elevated temperatures (no quantitative data available). In addition, the clamping device minimized evaporation from the chip as the polymer surface was covered with a transparent polycarbonate (PC) plate. Without the PC plate on top of the polymer chip the PCR chambers ran dry during the thermal cycling program.

**Optical setup**

Initial fluorescent signal intensity tests were conducted with epifluorescence microscopy (IX70; Olympus, Germany) of solutions containing either negative or positive samples that were compared to various fluorescein concentrations. Images were taken at 40x magnification (NA = 0.75) and 5 seconds acquisition time with an EMCCD camera (iXon, Andor Technologies, Ireland; gain = 0) and an appropriate filter set (excitation (470/40 nm), dichroic (495 nm) and emission (525/25 nm). For fluorescent monitoring of on-chip PCR a confocal laser scanning microscope was applied (LSM510, Zeiss, Germany). Imaging was conducted at 10x magnification with a 488 nm argon laser using the following microscope parameters:

- Optical setting 1: speed = 6 (15.2 s); zoom = 0.7; pinhole = 800; detection gain = 550; amplification offset = 0; amplification gain = 1.
- Optical setting 2: speed = 6 (15.2 s); zoom = 0.7; pinhole = 1000; detection gain = 650; amplification offset = 0; amplification gain = 1.
6.3.3 Results and discussion

Measurement modalities

Microfluidic RT-qPCR setups are currently based on custom-designed solutions for image acquisition, as the microfluidic devices are not fitted to conventional plate readers (212, 213, 341). These custom-designed or commercially available setups are, however, not compatible with the developed world-to-chip interface introduced in chapter 6.2. Thus, in a first step an optimal measurement setup had to be identified. An optimal setup is characterized by high sensitivity at low magnification to allow for simultaneous observation of multiple amplification chambers.

To retrieve a first quantification of the to-be-expected signal intensities bench-top generated samples were analyzed on a simple test chip. Total RNA derived negative (not amplified) and positive (PCR amplified) samples encoding the housekeeping gene *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) were flushed through a 20 µm high channel and the fluorescence was detected by epifluorescent microscopy. The obtained intensities were set into correlation to a fluorescein calibration (range 50 to 1000 nM) so that equivalent concentration units could be determined (Fig. 6.7A). These concentration units revealed an equivalent fluorescein concentration increase factor of 20.17 (positive/negative; positive sample = 277.41 nM, negative sample = 13.75 nM). Though the solutions containing varying analyte concentrations showed narrow intensity distributions and allowed to distinguish concentration differences below 50 nM fluorescein concentration equivalents, the absolute sample signal amplification factor was only 1.33 (Fig. 6.7B). Taking into account that these measurements were achieved at 40x magnification implies that the size of the PCR chambers and thereby the starting analyte amounts are limited using this setup. However, the results allude to significant improvement when the total detection volume is increased as is the case in the RT-qPCR chip device, i.e. a PCR chamber height of 100 µm instead of 20 µm. Further, the use of a confocal microscope setup would increase the signal to noise ratio thereby enabling lower magnifications.

Based on the first sample quantifications, a confocal microscope setup was evaluated for the suitability to monitor PCR induced fluorescence increase. This time, samples were directly flushed into the PCR chambers of the RT-qPCR chip device. Again, samples prior to and after PCR amplification (40 cycles on bench-top) were introduced into the chip and their fluorescent intensities were measured. Starting samples were derived by reverse transcription of 100 ng *Hprt* total RNA (equivalent to 10,000 cells, 10 pg per cell) and cDNA pre-amplification (15 cycles). To minimize analyte loss within the microfluidic device, BSA was added to a final concentration of 0.5 mg/ml (212, 345). Figure 6.8A depicts the relative fluorescence intensities of both samples (fluorescent micrographs and bar plot). Whereas the signal amplification in a conventional qPCR reader adds up to a factor of approximately 100 to 250 (cf. Fig. 6.9A), the same solutions measured on the confocal microscope setup differ by a factor of about 4.1 only, which emphasizes the sheer difference in the measurement modalities.
Fig. 6.7: A. Fluorescein concentration calibration (50, 100, 200, 250, 500 and 1000 nm) to indirectly quantify negative (not amplified) and positive (PCR amplified) Hprt (c)DNA samples generated in the bench-top approach and measured within a microfluidic channel. Arrows mark the data points whose intensity distributions are displayed in the histogram in B. Linear fit: slope = 1.6304, offset = 1275, $R^2 = 0.9959$. B. Histograms of the sample signal distributions are compared to the histograms of the closest fluorescein concentration intensities. The total signal amplification measured on chip and induced by bench-top PCR is only 1.33, which is not sufficient for a quantification of gene expression.

Fig. 6.8: A. Bench-top derived Hprt cDNA samples (prior to and after bench-top PCR) were flushed into the PCR chambers of the RT-qPCR chip device and signals were collected by confocal microscopy. Data was normalized to the sample prior to PCR amplification (cycle 0). Fluorescent micrographs depict the sample prior to PCR (left) and after PCR amplification (right) within the PCR chambers. $n = 5$ for each data set. B. Comparison of bench-top (micrograph left) and on-chip (right micrograph) derived PCR amplified samples. Data was normalized to the fluorescence of the sample solution prior to PCR. On-chip PCR revealed about 60% of the signal amplification provided by the bench-top PCR. $n$(bench-top) = 6, $n$(on-chip) = 2. Optical setting 1 was applied for both A and B. Scale bars: 200 µm.
For further characterization of the measurement setup, a first PCR amplification on-chip was conducted to evaluate the thermal cycling efficiency of the device in combination with the applied copper hot plates. A bench-top pre-amplified cDNA sample (same as before) was introduced into the PCR chambers of the RT-qPCR device and specific primers were introduced into the assay chambers. After mixing of the reagents the samples underwent 40 thermal cycles and the final fluorescence signal was measured. In parallel, a bench-top PCR was conducted starting with the identical cDNA sample solution. Figure 6.8B displays the on-chip amplification efficiency in correlation to the bench-top result. The amplification effectiveness for the initial on-chip trial is merely subjected to 40% less efficiency and therefore holds promising potential. A factor potentially affecting this loss in efficiency compared to the bench-top approach is the use of particular surface treatment molecules and surfactants that are wildly deployed in microfluidic PCR protocols and differ in composition as well as in concentrations each frequently adapted to the specific requirements (212, 341, 345, 346). The influence of the most commonly used agents was examined in a next step.

Surfactant additives and surface functionalization to prevent the loss of analytes

Besides the use of BSA to prevent the unspecific binding of agents to the channel walls, especially polysorbate 20 (Tween 20) has been chosen as a surfactant in microfluidic PCR protocols either alone (212, 346) or in combination with polyvinylpyrrolidone (PVP) (210). To prevent sticking of hydrophobic molecules to the polymer walls surface functionalization with PLL-g-PEG (poly(L-lysine)-g-poly(ethylene glycol) has further proven to be effective (347, 348). As in the bench-top approach the adhesion of molecules to the surface areas is comparably negligible so that the addition of surfactants is not common, the effect of the surfactants BSA and Tween 20 on the bench-top PCR was determined in a first step (Fig. 6.9A). About 13000 pg Hprt total RNA (~1300 cells) was applied to generate cDNA samples. BSA and Tween 20 led to very similar ct values in the bench-top qPCR suggesting similar end point fluorescent measures for on-chip cycling.

On-chip amplification was performed on the cDNA samples after mixture with the hprt primers for 40 cycles under varying combinations of surfactants and surface treatments all including Tween 20. For a better correlation of the experimental outcome and its relevance to the aimed for RT-qPCR of in vivo samples, this time RT and cDNA pre-amplification (bench-top) were conducted with LCM-derived osteocytes from cryosections (10 cells). The respective signal amplification factors are displayed in figure 6.9B and set into correlation to the on-chip measured fluorescent intensity of the bench-top derived PCR product (40 cycles, with Tween 20). Generally, the choice of other surfactants as BSA did not increase the amplification efficiency (cf. Fig. 6.8B). The exclusive addition of Tween 20 resulted in 56% signal amplification compared to the bench-top result (BSA: 60%). Of all on-chip PCR trials including Tween 20 the combination with PVP revealed the lowest amplification, whereas PLL-g-PEG in turn seemed to abolish the PVP-induced decrease. The use of PLL-g-PEG however is limited to microfluidic devices, which are either fully assembled prior to sample introduction or only those parts of the channel system can be treated that are not required right in the beginning. In the presented device these areas would exclusively cover the assay lines, as the reverse transcription of the collected samples has to be initiated as early as possible by necessity (see sub-chapter Time critical mRNA stability).
Fig. 6.9: A. Bench-top results of qPCR under the influence of 0.1% Tween 20 and 0.5 mg/ml BSA. B. Signal amplification derived by bench-top and microfluidic PCR (40 cycles) of cDNA samples (hp rt) measured on-chip. Top to bottom: n = 6, 4, 4, 4. Final data (cycle 40) was normalized to the initial fluorescence value (cycle 0) and statistical significance was calculated using the two-sample Welch test. Optical setting 1 was applied for on-chip measurements.

On-chip PCR of total RNA probes

The characterization of the RT-qPCR protocol was first tested using total RNA and the gene expression assay for the housekeeping gene Hprt. The protocols were run for the bench-top and the on-chip approach as described in the methods section. BSA was added to a final concentration of 0.5 mg/ml for the on-chip experiment to limit analyte loss. Figure 6.10 depicts the qPCR reactions for 20 cycles on-chip and 40 cycles on bench-top after RT and pre-amplification. Unfortunately, valve malfunctions during the on-chip experiment did not allow for fluid transfer from the sample chambers via the mixing chambers into the PCR chambers. Hence, RT, pre-amplification and qPCR were all run in the sample chamber, whereby partial fluid transfer into the mixing chamber allowed the addition of the required PCR buffer mixes already including Hprt specific primers. Further, thermal cycling could not be performed longer than 20 PCR cycles due to fluid leakage. Total RNA starting amount was again 100 ng in each approach (~10,000 cells). PCR amplification on-chip was characterized by the absence of a lag phase, however, same time by a strong decrease in amplification after only few cycles (Fig. 6.10).

Whether the reason(s) (besides the inaccurate mixing ratios) are due to inefficient thermal cycling (manual chip transfer), analyte loss or low sample starting concentration remains to be elucidated. The manual handling of the device is additionally prone to bring along measurement inaccuracies, as the relocation of the device onto the confocal microscope table requires re-adjustment of the focus, which in combination with confocal microscopy leads to strong fluorescence signal aberrations. Nevertheless, first indications for the functionality of the RT-qPCR microfluidic device were provided. The limitations to be addressed next are the reaction sensitivity (lower starting RNA concentration) and the microfluidic device reliability (valve malfunctioning). Notably, adaptation of commonly applied fabrication protocols (213, 341, 345) would not improve the device reliability as those chip devices are all fully assembled prior to sample introduction. In our case the fabrication protocol demands full assembly only after sample capture and therefore requires a bonding strategy that does not interfere with the sample transfer and valve function.
Fig. 6.10: Comparison between the RT-qPCR trials of total RNA (expression assay for Hprt) conducted on-chip (closed black diamonds) and on bench-top (open grey circles). The basal intensity on chip is higher as a reference dye was included and the measurement was conducted with the confocal setup (optical setting 2). n(on-chip) = 3 chambers and n(bench-top) = 1.

**Time critical mRNA stability**

All time periods from cell dissection to chip assembly and thermal cycling were documented. The timeline for the whole process is listed in table 6.7. Although mixing times on-chip contributed to a large extend to the total processing time, analyte stability can be presumed after reverse transcription and analyte loss can most likely be neglected due to the use of surfactants (212, 213, 341). The environmental conditions for the osteocytes within the cryosection placed under the LCM microscope is on the contrary time critical as mRNA has been shown to degrade fast. Earlier studies reported degradation rates of 99% for mRNA encoding β-actin within 20 minutes after thawing of untreated cryosections (349). The time duration required to fill all twelve cell capture chambers took about 2 hours and thus no successful microfluidic RT-qPCR of dissected osteocytes was completed for single as well as for populations of osteocytes. During this sample collection period the mRNA most probably degraded down to a concentration not adequate for reliable reverse transcription and amplification. Certainly, the laser induced heat will lead to further sample damage, however, as shown in first reports on RT-qPCR of the same housekeeping gene (β-actin), adequate LCM protocols are capable of minimizing sample loss during the dissection process (334). To circumvent mRNA degradation cryosections were handled throughout LCM for bench-top sampling for 30 minutes only. Degradation in the samples was generally slower than reported from Murakami et al. (349) as the cryosections underwent dehydration in methanol prior to LCM (350). This allowed the prolonged sampling time on the LCM microscope, which however was still significantly shorter than the time required to fill the chip device.
Tab. 6.7: Timeline for sample dissection and microfluidic and bench-top RT-qPCR.

<table>
<thead>
<tr>
<th>Task</th>
<th>On-chip</th>
<th>Bench-top</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissection and transfer (LCM)</td>
<td>2 h</td>
<td>10 min*</td>
</tr>
<tr>
<td>Chip assembly and bonding</td>
<td>15 min</td>
<td>-</td>
</tr>
<tr>
<td>Implementing connections for control and fluid layer</td>
<td>15 min</td>
<td>-</td>
</tr>
<tr>
<td>Flushing RT chambers</td>
<td>5 min</td>
<td>-</td>
</tr>
<tr>
<td>RT and pre-amplification</td>
<td>85 min</td>
<td>81 min</td>
</tr>
<tr>
<td>Addition of PCR buffer, transfer in mixing chamber and complete mixing</td>
<td>2 h</td>
<td>20 min</td>
</tr>
<tr>
<td>Distribute samples in PCR chambers</td>
<td>5 min</td>
<td>-</td>
</tr>
<tr>
<td>Addition of assay mix and mixture of assay and PCR chambers</td>
<td>2 h</td>
<td>-</td>
</tr>
<tr>
<td>PCR cycling</td>
<td>1.5 h</td>
<td>62 min</td>
</tr>
<tr>
<td>Total endurance</td>
<td>~9.5 h</td>
<td>~4.8 h</td>
</tr>
</tbody>
</table>

* LCM for the bench-top approach took about 10 minutes for each sample. For comparison the time needed for 12 sampling trials was included into the total endurance time.

6.3.4 Conclusions and outlook

A multi-layered PDMS microfluidic chip device for the application of RT-qPCR was developed and first results using total RNA samples could be obtained. Although the chip device provides promising properties, the amplification results so far only showed qualitative character. Impairments were mainly identified in the manual operation of thermal cycling and imaging, which results in temperature inaccuracy as well as in focusing differences. Thus, thermal cycling and imaging on-site, i.e. fixed on the microscope stage, are crucial to circumvent loss in amplification yield as well as optical monitoring inaccuracy. Solutions may be the use of a flat bed thermocycler and imaging with a stereomicroscope or the development of thermal control by the combination of electrodes for heating (e.g. indium tin oxide electrodes) and cooling by Peltier-elements. In the latter option, the electrode pattern would need to be arranged in a grid, whereby it circumvents the amplification chambers to not limit the detected signal. This arrangement still allows the signal detection with confocal as well as inverse wide field microscopy thereby providing higher sensitivity than provided by a stereomicroscope.

Besides the initial results derived from the processing of total RNA probes on a manually run platform, the amplification of mRNA from harvested osteocytes was not successful. Hereby the RT-qPCR protocol was most likely not the limiting factor but rather the stability of the mRNA itself. Due to this fact the most critical workflow step is the cell dissection and transfer that currently take about 2 hours before the samples can be further processed on-chip. Either this time period can be substantially shortened in future protocols or other strategies may have to be considered, such as the in situ reverse transcription of mRNA prior to cell dissection (345, 351). After in situ transcription of mRNA to stable cDNA, the duration of the dissection and transfer process would no longer be time critical.
Acknowledgements

Initiation of the project and first technical interface testing by Duncan J. Webster is gratefully acknowledged. I also thank Andreas J. Trüssel for proofreading this chapter.
World-to-chip interface
Chapter 7

Concluding discussions and outlook

The main objective of this thesis was the development, characterization, and successful application of microfluidic analysis platforms that facilitate the investigation of mechanotransduction processes at the cellular level. A crucial step for the establishment of *in vitro* real time analysis of molecular mechanotransduction events in murine myoblasts was the engineering and characterization of a stage perfusion incubator that hosts a specifically tailored microfluidic chip. This platform was then utilized to investigate molecular mechanotransduction as a result of the activation of mechanosensitive TRP channels in murine myoblasts. After establishing of a strategy for mechanical stimulation as well as for cell response readout, the involvement and identification of particular TRP channels in the mechano-response under varying conditions was successfully achieved. The second presented platform served two principle objectives: (i) a world-to-chip interface that can be used to selectively transfer cells out of *in vivo* samples into a microfluidic system and (ii) the cell content analysis of the dissected cells. This chapter summarizes the achievements of each individual project and discusses their implications in their engineering and biological perspective, respectively.


7.1 Concluding discussions

Construction and characterization of a stage perfusion incubator compatible to standard PDMS microfluidic devices

The first technical task emerged from the cell culture requirements for the investigation of functional mechanosensitive TRP channels in murine myoblasts. As discussed in chapter 4, various ways exist to culture cells in microfluidic chips, which is dependent on the tight control of nutrient supply, temperature, and pH. Nutrient supply is technically the easiest achievable of these vital factors, since the nutrients are part of the medium. Still, various perfusion strategies for medium supply exist that upon change in perfusion script simultaneously alter medium composition in the cell environment as well as influence cell-to-cell communication by secreted signaling factors (173, 281). For the culture of C2C12 myoblasts a periodic delivery of slow medium pulses provided optimal conditions for growth and viability. Importantly, the establishment of a perfusion script should not interfere with the addressed biological objective. As perfusion in microfluidic dimensions induces friction at the solid-to-liquid interface, cells attached to the channel surface undergo shear forces. These shear forces for instance activate mechanosensitive TRP channels (296, 297), and thus careful development of a suitable perfusion script is crucial not to compromise the underlying experiment.

At first glance, temperature regulation of microfluidic chip cultures may be regarded as a trivial matter of additional heating devices such as resistance heaters or hotplates and is therefore only briefly mentioned in reviews summarizing on-chip cell culture (173). However, resistance heaters generate electromagnetic fields, which can possibly interfere with the objective of the experiment. That is, the gating of mechanosensitive channels cannot only be altered by mechanical actuation or chemical modulators (cf. chapter 5), but pulse electromagnetic fields (PEMF) have been proven to activate these channels as well (A. Franco-Obregón; unpublished information). A temperature control using resistance heaters thus involved the theoretical risk of an induction of interfering electromagnetic fields. To prevent this risk of interference, a configuration was chosen that limited the induced electromagnetic fields to 10 µT, which is about 200-fold less than the field strengths required for the PEMF induced channel activation.

Appropriate medium pH must be established to ensure maintenance of successful cell culture. While atmospheric CO\textsubscript{2} levels are utilized to regulate media pH in conventional cell culture incubators, microfluidic systems circumvent medium pH regulation by CO\textsubscript{2} since it often goes along with highly specialized and complicated chip designs (279, 280). As a matter of fact, pH control in microfluidic cell cultures commonly relies on the use of buffers, which may comprise biological limitations (276). In particular, buffers such as HEPES also modulate the gating of mechanosensitive channels (56, 277). Thus, the only way to achieve effective pH regulation for microfluidic long-term culture was to control CO\textsubscript{2} levels within the system. Constant CO\textsubscript{2} levels were maintained in the surrounding atmosphere that in combination with the gas permeability of PDMS enabled CO\textsubscript{2} and oxygen permeation through the chip material into the fluidic channels. Gas supply was realized by the introduction of a constant gas mix feed (7% CO\textsubscript{2}, synthetic air). As the gas was not pre-saturated with water, liquid reservoirs inside the incubation chamber were added for 100% humidity of the atmosphere. The latter is particularly important for PDMS microfluidic systems as evaporation of the small volumes through the polymer occurs fast even at moderate elevated temperatures.
Controlling the environmental temperature and medium pH as described above permitted the successful on-chip perfusion culture of C2C12 murine myoblasts and HEK 293 cells for two and five days, respectively. Growth rates as well as viability rates were both concurrent with those observed in conventional flask cultures, thus proving the suitability of the developed incubation system for the pursued mechanobiological studies. Moreover and from a very practical perspective, the established system allows for the robust fixation of a variety of supply tubing and the chip device itself (Fig. 7.1). Principally, due to this robustness as well as its simple peripheral devices, the incubation system is portable as long as CO₂ supply is granted. Although over the last three years stage incubation systems with perfusion option have become available for purchase, more specialized microfluidic application requirements, such as variable tubing supply ports, still rely on custom-design and are thus rather expensive compared to the presented solution. In addition, commercially available systems are generally specified for one specific microscope stage type. On the contrary, the presented solution is compatible with any x-y microscope stage of conventional inverse microscopes, thus further expanding possible applicability. In summary, the developed stage perfusion incubation system serves well for the culture of mammalian cells in PDMS-based microfluidic chips and provides a practical and versatile tool for microfluidics laboratories.

**Microfluidic analysis of molecular mechanotransduction in murine myoblasts**

Mechanosensitive cation channels of the TRP family have been identified as key components in mechanotransduction cascades (45). In myogenesis these channels play key roles in cellular development by transducing mechanical forces impinging onto the cells into calcium entry that for example regulates decisions on proliferation and differentiation (10). The characterization of TRP channels has largely been permitted by the patch clamp technology, which fostered insights into channel identification as well as precise mechanistic descriptions of channel function and regulation (36, 37, 83). Besides its outstanding accomplishments, however, the patch clamp technology faces inherent challenges that are partly related to its invasiveness as discussed in chapter 1.1.3 and 5. In addition, although the measurement of trans-sarcolemma potential or current reveals information on instant gating activity and thereby on calcium entry, calcium homeostasis in muscle cells is much more intertwined. The regulation of cytosolic calcium levels further includes calcium entry by calcium induced calcium release (CICR) from the intracellular stores in the sarcoplasmic reticulum (38, 40, 41) as well as the activation of store-operated channels (SOC) (39). Mere monitoring of calcium entry from the extracellular space cannot
assess both of these latter regulatory pathways. Furthermore, cell-to-cell communications and the impact of trophic factors, i.e. cell environmental compounds, on TRP channel recruitment are to some extend difficult to address and interpret using the patch clamp. The objective of this study thus was to monitor whole cell responses depicting external calcium entry as well as release from intracellular stores that are not limited in cell-to-cell communication and the effect of trophic factors. Still, the potential to simultaneously decipher the respective contributions of diverse TRP channels was required to persist. To achieve this goal, a microfluidic strategy was developed that allowed for non-invasive precise stimulation and response readout of cell responses from small communities of cells.

The basis of the specifically developed microfluidic platform was a double-layered chip design that enabled myoblast migration at controlled cell confluenes during on-chip culture as well as induction of accurate shear rates (chapter 4 and 5, Fig. 7.1). By implementation of a control layer, with which cell capture structures were lowered into the cell culture layer on demand, a rectangular channel geometry was guaranteed after cell seeding. Attached cells could then freely migrate within their 2D culture, which would not be possible in case conventional permanent cell capture structures were chosen for cell seeding. Consistent channel geometry is additionally of fundamental significance for a steady fluid flow profile. Since the cell capture structures did not remain in the culture chamber for perfusion culture and mechanical stimulation, the flow profile was left ideal for accurate creation of wall shear rates and constant mechanical cell stimulation.

Real-time monitoring of changes in cytosolic calcium increments as a response to these applied shear rates was accomplished with a calcium sensitive fluorescent dye in conjunction with epifluorescence microscopy. Both, the mechanical stimulation as well as readout of the cell responses were non-invasive thereby matching the initially set criteria. Although the measurement acuity did not attain the sensitivity scored by the patch clamp, the readout of the whole cell mechano-response encompassed not merely trans-membrane channel gating, but at the same time CICR and the potential activation of SOCs. The recording paradigm disclosed TRP channel specific characteristics, namely (i) increasing cytosolic calcium increments upon mechanical stimulation and cell recovery under static conditions, (ii) attenuated cytosolic calcium increments upon repeated mechanical actuation (92, 303), and (iii) mechanically-mediated response intensities graded by the magnitude of the shear rate (37, 92, 303). The established strategy additionally reflected culture time-dependent changes in mechanosensitivity reported in earlier studies, whereby the magnitude of calcium entry closely paralleled TRP channel expression levels that changed during time in culture (72). The key findings unveiled by this study demonstrated that TRP channel recruitment is intrinsically dependent on serum levels and extracellular matrix (ECM) composition. Combinatorial examination of these extraneous factors (serum concentration, ECM composition, time in culture) and channel expression revealed a predominant role for TRPV2 in shear stress mediated mechanotransduction. Pharmacological discrimination of the three widely accepted mechanosensitive TRP channels TRPC1, TRPV2 and TRPM7 substantiated a central role of TRPV2 in the calcium response to fluid shear stress, which has so far not been reported.

As up-to-date TRPC1 has been shown to largely account for mechanically-mediated calcium entry in myoblast cells, the demonstrated role for TRPV2 is rather fundamental under the following considerations. (i) TRPV2 has been ascribed functional roles in pathophysiological calcium entry in mdx muscle (68, 70), however, its relevance to calcium entry in normal
myogenesis has not been reported. This indicates a more complex role of TRPV2 in myogenesis than previously anticipated and may possibly lead to further important clues, e.g. on how and why TRPV2 loses its vital gating regulation in mdx muscle. (ii) Conventional tissue engineering studies on cellular mechanosensitivity commonly use serum level ranges, which showed to attenuate TRPV2 activity (10 - 20% serum) as well as apply fibronectin as surface treatment to support cell-substrate adhesion, which substantially extenuated the magnitude and the dynamic cell response to mechanical force. Cell mechanobiological studies are thus obliged to evaluate basic cell culture protocols for their compatibility with their study objective, otherwise the cell mechano-response might be misinterpreted. Re-thinking of established cell culture paradigms may help to decipher fundamental mechanotransduction events and identify their relevance for cell developmental decisions. Another observation underlines this highly complex coordination in cellular mechanotransduction. The monitored mechanical phenomena were contingent on the cell developmental stage, which in turn even temporally reinforced the changes in mechanosensitivity imposed by serum and ECM. The interconnection of molecular mechanotransduction and extraneous factors is here widened by a very dynamic factor, i.e. time in culture, which yields unequal outcomes between different cell types depending on the respective proliferation rates. Concluding from these remarks, the role of TRPV2 could not have been deciphered under conventionally applied culture conditions. The presented microfluidic platform in combination with the established stimulation and recording paradigm in contrary allowed for the concerted investigation of TRP channel mediated mechanotransduction processes under an unprecedented experimental variability in culture environmental conditions.

Besides the proof for TRPV2 channel recruitment under shear stress in myoblasts, the results further suggest mechanistic aspects of TRPV2 channel activation under the described conditions. TRPC1 has been shown to gate under direct association with the dystrophin glycoprotein complex (DGC), which is known to mediate outer mechanical stretch via binding to laminin in the ECM (51). As laminin showed to fortify cell mechanical-responses under conditions favoring TRPV2 activity (Fig. 5.3 and 5.4), the reported data suggest that TRPV2 also undergoes this association in the early stage of myogenesis (Fig. 5.3, Fig. 7.2). The indication for TRPV2 association to the DGC complex may partly contribute to explain failures in channel gating in dystrophic muscle, where the DGC complex lacks its protein content that associates with the TRP channel, i.e. dystrophin. Though TRPV2 has been identified to be partly responsible for the elevated calcium entry in mdx myotubes (68, 70), a mechanistic link to the DGC complex and, hence, dystrophin has so far not been demonstrated.

The presented findings not only provide valuable leads for future studies about myogenesis to follow up on, they also substantiate the intricate roles of mechanotransduction processes and environmental conditions. Cellular mechanotransduction is an essential determinant of stem cell fate decisions and many studies have unintentionally misprized the direct relation between mechanosensitivity and trophic factors: established protocol details, such as the use of high serum contents, were left unquestioned. Just few years back, mechanobiology was treated rather as an epiphenomenon than a fundamental factor regulating cell fate (4). Today, the scientific community is indeed aware of the implications mechanotransduction processes impart on developmental decisions, although many of them still remain poorly understood (15, 20). The presented study now attracts the attention to multi-modular correlations between molecular mechanotransduction and modifying factors, which may be affected by conventional cell culture paradigms.
Fig. 7.2: Schematic representation of the proposed mechanistic TRPV2 activation in myoblasts under fluid flow induced shear forces. TRPV2 is recruited to the sarcolemma under shear force and stretch-activated through direct association with the DGC complex, which links to external laminin. Calcium entry primes calcium release from the intracellular stores (SR; sarcoplasmic reticulum). The increasing cytosolic calcium levels activate phospholipase C (PLC), which in turn results in PiP$_2$ depletion within the inner membrane leaf consequently leading to reduced channel activity.

World-to-chip strategy for cell analysis of in vivo models

While in vitro studies can be performed under fairly controlled conditions at the single cell level, the response of individual cells stimulated in vivo is very difficult to access. Current gene expression analyses of cells derived from in vivo samples are tedious and lack the ability to target multiple genes of interest, when handled in the bench-top approach. Integrated microfluidics have the potential to automate analytical programs as well as multiplex analytical targets. The development of such a µTAS first had to overcome the gap between in vivo samples, which bear crucial local sample information besides the cells themselves, and a closed microfluidic environment. The developed strategy thus envisaged an open channel system into which dissected cells can be transferred and that after completed sample collection can be closed and then addressed same as a conventional microfluidic chip.

The experimental strategy was based on already existing methods for the characterization of mechanically-regulated bone formation and resorption processes in mouse caudal vertebrae (13).
Concluding discussions and outlook

An *in vivo* mouse model was applied for the transient and dynamic mechanical loading of bone (335). The hereby induced highly localized changes in bone mass were correlated to local strain energy densities within the bone architecture derived by finite element analyses of micro-computed tomography (μCT) images (14, 337) (Fig. 7.3A). Based on these precise mappings, single cells or cell clusters of interest were dissected out of the bone matrix by laser capture microdissection (LCM) (Fig. 7.3B). Current methods would continue with manual handling of the sample and the analysis of few genes of interest (334, 336). Contrariwise, the developed platform facilitates the capture of the dissected cells within capture chambers that are part of an open microfluidic channel layer. After sample collection in the capture chambers, the open PDMS chip was closed with a second layer, i.e. the control layer. The control layer enabled subsequent valve actuation within the completed chip device thereby controlling fluid supply for sample preparation and partitioning of each cell sample individually (Fig. 7.3C). Sample processing was planned to contain all necessary steps for RT-qPCR including the targeting of up to eight different genes. As the experimental strategy processes each cell sample separately, the local information of the initially generated strain energy density maps of the bone cryosections are preserved and retrieved gene expression patterns can be correlated to the magnitude of mechanical load (Fig. 7.3D).

The first task of this ambitious project was to establish the link between the *in vivo* sample and the μTAS (chapter 6.1). By careful design of the capture chambers and the optimization and characterization of the LCM process, this gap could be overcome. The capture chamber design was bound to account for the LCM accuracy as well as the final volume within which the cell sample was treated for further analysis after assembling the final chip. Initial evaluations of the cell integrity after LCM indicated cell sample integrity bearing well-preserved DNA/RNA content. To our knowledge the developed strategy presents the first experimental solution that allows for the analysis of *in vivo* derived cell samples in microfluidic systems. It is thus of high interest to the scientific community as the experimental workload can be substantially reduced using automated μTAS, which furthermore have proven to provide higher sensitivity and less sample loss than conventional bench-top approaches (127, 159).

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**Fig. 7.3:** Strategy to access the mechanically-induced gene expression of osteocytes: A. μCT-derived finite element analyses provide strain energy density values of the bone section under mechanical loading that are matched with areas of newly formed or resorbed bone. B. Sample dissection from the bone cryosection (left) and transfer of the cell(s) of interest into the microfluidic device (right) is accomplished by LCM. Scale bars: 100 μm. C. The developed μTAS allows for the isolated capture and subsequent analysis of the osteocytes. Scale bar: 1 mm. D. Data can be linked back to the local environment of the cell within the bone matrix and gene expression levels can be correlated with levels of mechanical load.
Already established protocols (336, 352) were adapted for the microfluidic RT-qPCR (350) and tested for their compatibility with bone samples. Particular focus was laid on (i) chip functionality, (ii) optical readout modality and performance, and (iii) surface treatment and detergent supplementation to prevent probe and analyte loss. All derived data was compared with bench-top results. Furthermore, preliminary studies with total RNA probes were conducted to characterize chip-processing performance. Initial characterization of the chip device and the optical measurement method showed promising results regarding chip function and detection signal to noise ratio. The processing of total RNA samples provided first (RT-)PCR results, however without quantification of the starting RNA probe. In comparison, neither the processing of total RNA probes or whole cell samples matched the performance of the bench-top handling. The reasons identified were first that the elementary temperature cycling was conducted manually next to the microscope (different hot plates). This lead to temperature cycling inaccuracies as well as inevitably entailed inaccurate data points since the focus settings of the confocal microscope were hardly possible to be re-matched after reinsertion of the chip device into the microscope stage after each temperature cycling. Second, RNA degradation in the cryosections compromised effective RNA handling on-chip due to the long cell collection period during LCM (cf. Tab. 6.7). Potential solutions to these challenges are listed in the outlook section.

Notably, one commercially available microfluidic system for multiplexed qPCR exists (company Fluidigm, CA, USA). However, compared to our envisaged platform this system still has the drawback that it can only handle pre-processed samples, i.e. RT and pre-amplification of the cDNA have to be conducted in the conventional bench-top approach. A solution that incorporates all process steps in one system (RT-qPCR) is therefore of high relevance to the scientific field.
7.2 Outlook

**Microfluidic analysis of TRP channels and their implications on mechanotransduction**

Though the established microfluidic strategy to determine molecular mechanotransduction events on the basis of fluorescent calcium imaging lead to important novel findings regarding the particular roles of distinct TRP channels in the early stage of myogenesis, further improvement of the platform is necessary. First, the improvement of the data readout and the generation of bigger data sets are needed. Automation of the established platform would provide vast increasing numbers of cell readouts generating population data at single cell resolution, which would never be achievable using the patch clamp. This would lead to higher significance of the retrieved data as well as would enable the evaluation of cell-to-cell communication, for instance by correlating the cell confluence to individual levels of cell-mechanosensitivity. Although the present solution allows for the monitoring of small communities of cells, the resulting cell numbers per experiment are still not sufficient to conduct cell population analyses. Second, to increase the platform’s sensitivity, the optical detection should be changed to a confocal microscope setup. This will limit signal-to-noise fluctuations that have shown to partially influence the epifluorescence readout and will thereby generate robust time-lapse cell data. As a result, the improved signal-to-noise ratio would allow for a better discrimination of cell-to-cell variances from the changes evoked by the experimental conditions. The increased sensitivity will facilitate monitoring of cell responses at shorter time scales, which permits a more precise determination of channel gating kinetics. It may also enable the determination of spatiotemporal changes in calcium increments, which may contribute to a more precise description of cell responses to fluid shear stress.

Calcium signaling in skeletal muscle does not necessarily occur uniformly across the cell. Evidence exists that besides the general cytosolic calcium increase upon mechanical stimulation, spatially- and temporally-defined high calcium increments induced by calcium release from the intracellular stores prime the activity of SOCs (120). These highly localized calcium increments, i.e. calcium sparks, have been attributed to calcium release by the ryanodine receptors (RyR) of the SR, and can be further controlled by mechanosensitive TRP channel gating (CICR). First initial studies on the monitoring of spark events in C2C12 myoblasts have been conducted using the identical setup as described in chapter 5. Figure 7.4 depicts characteristic calcium spark events upon fluid flow induced shear stress. Although the geometry (area) and the intensity of these calcium events were in concordance with literature (119, 121), they seldom occurred. This may be due to two aspects. First, the spark monitoring protocols were developed under high serum concentrations, and hence, prior to the finding that high serum concentrations strongly attenuate TRP channel activity. Consequently, the incidence of a potential event of a calcium spark primed by CICR may be attenuated as well. Second, calcium sparks are commonly detected with confocal microscopy (119). Since the presented setup applied epifluorescence microscopy, many spark events might have been lost during monitoring due to the lower sensitivity. Future studies on calcium spark activity in myoblast cells could either start with the identical setup and under low serum concentrations or using confocal microscopy. Successfully obtained results could be correlated to the results retrieved on global calcium increments thereby potentially providing indications on the relative contributions of extracellular calcium entry (TRP channels) as well as calcium release (RyR) on the global change in calcium concentration.
Fig. 7.4: Example of a calcium spark-signaling event under fluid flow actuation. A. Bright field micrograph of a skeletal myoblast cell under fluid flow conditions with red dots marking the local spark events. B. Fluorescent micrograph from image sequence showing bright signals of simultaneously occurring calcium-binding events. C. A 3D graph of the calcium-signaling events depicts reasonable geometrical characteristics and intensities. Scale bars: 10 µm. A MATLAB program for the detection of calcium sparks is listed in the appendix (A.4.2).

Besides the improvements of the presented experimental paradigm, microfluidic technology offers great potential to increase the information density per experiment (131). For this, changes in chip design can be considered to achieve monitoring of different conditions in parallel. A chip design including culture chambers of varying heights would for instance facilitate force-dependent investigations on channel activation within a cell population at once. Next, the introduction of separated fluid systems in one chip has additionally the potential to supply different agents during mechanical stimulation in one experiment and monitor their effects simultaneously. Since TRP channels are exciting targets for drug development (48), this could be applied for high throughput pharmacological screening, whereby the effects of different channel (ant)agonists or different concentrations of the same channel modulator can be monitored during a single experiment for large cell numbers. Not only the raising of EC50 values as a function of whole cell responses could be retrieved, same time the discrimination of contributions from different TRP channels would hereby be highly encouraged. For instance, a more precise description of TRPV2 function in myoblasts under mechanical input could be achieved this way.

The versatility of microfabrication technology permits studying cell mechano-responses under changing substrate properties. In the presented study cell mechano-responses have been retrieved from cells plated on glass. Changing the young’s modulus of the substrate will provide valuable insights into TRP channel mediated mechanotransduction events as cells have shown to alter their mechanosensitivity and in turn their developmental fate based on the substrate stiffness (241). This can be achieved by seeding cells onto a PDMS surface instead of onto glass. PDMS could be fabricated with different stiffnesses and surface functionalization with ECM proteins could be conducted by micro-contact printing. Micro-contact printing would then over and above allow for the printing of specific protein patterns, same on PDMS as well as on glass, to directly compare the effect of different ECM compositions on cell mechanotransduction. Also, dynamic changes in substrate stiffness, induced for example by actuated membranes, may account for a better description of TRP channel gating and its relevance in governing cell migration, proliferation (353), and differentiation.
World-to-chip interface

The presented world-to-chip strategy outlined numerous opportunities for further research focus. A first step to encounter the discussed challenges would be continuing integration of temperature control as well as revaluation of an automated optical readout that both will certainly pave the way for an improved platform performance. The integration and automation will reveal (i) accurate temperature cycling, which is crucial for effective RT-qPCR, and (ii) accurate signal readout, which will allow for the quantification of gene expression based on the microscopy images. Additionally, these improvements will shorten the total processing time as well as considerably limit experimental workload. Mandatory for a completely autonomous integrated microfluidics system is moreover the entire automation of fluid supply and valve control.

The processing of bone cells on-chip exhibits more potential complications than cells from a conventional suspension culture due to the calcified bone matrix residing part of the sample (350). However, recent optimizations in the cryosection preparation and bench-top RT-qPCR protocols were able to generate highly quantitative gene expression readouts (350). Including these protocol optimizations in the microfluidic protocol would certainly improve the results of the microfluidic process. A time-protocol adaptation, with which the time between cryosection thawing and RT processing is significantly shortened, will further result in higher RNA preservation. For this a fast cell detection algorithm combined with a fast LCM procedure is necessary. The lag time from cryosection thawing until initiation of the RT must hereby not exceed 30 minutes (349, 350). An alternative option to guarantee the safe retrieval of the expression levels could be the in situ reverse transcription and subsequent transfer into the chip device (345, 351). This way, the time limitation of the LCM procedure would not be critical any more and the microfluidic protocol would additionally be simplified since the on-chip RT process step is omitted. Besides the project-specific future perspectives for the analysis of bone samples, the investigation of other tissue samples may assist in the future development of the gap-closing platform as well as demonstrate its extensive applicability.

Upon successful establishment of the RT-qPCR chip, the entire interface could be modified for the investigation of in vitro stimulated myoblast cells. The modifications would require the implementation of cell culture chambers instead of the cell capture chambers of the presented design to culture and mechanically stimulate C2C12 myoblasts under varying conditions. Afterwards, the cell culture chambers would be isolated from each other by valves to allow for the RT-qPCR of small myoblast cell communities. The acquired TRP channel expression levels could thus be directly attributed to the culture and stimulation conditions. Without modifications of the RT-qPCR chip interface, the analysis of muscle cells dissected from in vivo samples of mouse models may be rendered possible. As a prerequisite, these studies demand the careful adaptation of the tissue preparation protocols for LCM. This way detailed insights into the development of individual cells embedded in muscle tissue could be retrieved and associated to local strain energy densities.
References
References


References


References


Appendix
## A.1 Materials

The following table lists the commonly used materials and the respective suppliers in alphabetical order. All reagents and substances were used as delivered unless otherwise noted (see results sections for details).

<table>
<thead>
<tr>
<th>Chemical substance / reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-ethenylpyrrolidine-2-one (polyvinylpyrrolidone, PVP)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>1H,1H,2H,2H-perfluorodecyl-dimethylchloro-silane</td>
<td>ABCR</td>
</tr>
<tr>
<td>2-Aminoethyl diphenylborinate (2-APB)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>3-methacloxypropyltrimethoxysilane (MEMO)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS)</td>
<td>Acros Organics</td>
</tr>
<tr>
<td>Aminoethylaminopropyltrimethoxysilane (AEAPS)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>AZ-9260 and developer</td>
<td>MicroChem, Newton, MA</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA, fraction V)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Chloroform (analytical grade)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (cell culture grade) (DMSO)</td>
<td>Axonlab</td>
</tr>
<tr>
<td>Dulbecco’s modified eagle medium (all modifications) (DMEM)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Ethanol (analytical grade)</td>
<td>Fluka / Scharlau</td>
</tr>
<tr>
<td>Ethylene diamine tetraacetic acid (EDTA)</td>
<td>Merck</td>
</tr>
<tr>
<td>Ethylene glycol tetraacetic acid (EGTA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>F-8888 Component D (fluorescent particles)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Fibronectin (FN)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Fluo-4 AM</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Fluo-4 pentapotassium salt</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>Fluka</td>
</tr>
<tr>
<td>Foetal bovine serum (FBS)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Hank’s balanced salt solution (HBSS, with Ca(^{2+}), Mg(^{2+}))</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Hexamethylene disiloxane (HMDS)</td>
<td>Merck</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Merck</td>
</tr>
<tr>
<td>Isopropanol (analytical grade)</td>
<td>Scharlau</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Laminin (LN)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Methanol (HPLC grade)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
<td>PAA</td>
</tr>
<tr>
<td>Penicillin - streptomycin</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Penicillin - streptomycin - L-glutamine</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Pleuronic F-127</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Poly(dimethyl siloxane) (PDMS)</td>
<td>Dow Corning</td>
</tr>
<tr>
<td>Poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG)</td>
<td>SuSoS AG, Dübendorf, CH</td>
</tr>
<tr>
<td>Poly(vinyl alcohol) (PVA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Primers for TRPC1, TRPV2 and TRPM7</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Recovery™- cell culture freezing medium</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Retinoic acid (98% HPLC) (RA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>RNaseZAP®</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Ruthenium red (technical grade) (RR)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>SKF-96365</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium sulfate (anhydrous, analytical grade)</td>
<td>Merck</td>
</tr>
<tr>
<td>SU-8 2015 / 2050 and developer</td>
<td>MicroChem, Newton, MA</td>
</tr>
<tr>
<td>Synthetic air – carbon dioxide mix</td>
<td>Pangas</td>
</tr>
<tr>
<td>TaqMan PCR Components</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Total Hprt RNA</td>
<td>TaqMan, Thermo Scientific</td>
</tr>
<tr>
<td>TRIZol</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>TrypLE Express</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
A.2 Fluo-4 bleaching controls

**Fig. A.2.1:** Comparison of Fluo-4 (1 µM) photobleaching in HBSS buffer solution (1.26 mM Ca\(^{2+}\)) under permanent exposure to fluorescence excitation of varying intensities. Each single data set depicts representative curves under the respective conditions. No photobleaching could be detected even after 10 min of permanent exposure with low excitation intensity (not shown). Low intensity excitation was applied in cell experiments and accounts for 12% of high intensity excitation.

**Fig. A.2.2:** Relative Fluo-4 (1 µM) fluorescence signal intensity in HBSS buffer solution (1.26 mM Ca\(^{2+}\)) as a function of EGTA concentration. Depicted are mean values ± SD of three independent measurements. The results clearly substantiate the calcium dependent signal specificity of the control experiment.
A.3 Chip designs

Chip designs for *in vitro* cell analysis

**Fig. A.3.1:** A. Fluidic CAD design used for the culture of Human Embryonic Kidney cells (HEK-293). B. Fluidic CAD design used as basis for the culture of C2C12 murine myoblasts. This channel design was mostly applied in conjunction with the control layer depicted in figure A.3.2. Channel heights were 100 µm (A) and 40 µm (B), both SU-8. Scale bars (A, B): 2 mm.

**Fig. A.3.2:** A. Close-up of the CAD derived cell capture structures as part of the control layer. B. Overlay of the two-layered CAD chip design applied in the capture, culture und mechanical stimulation of C2C12 murine myoblasts. The used fluid layer is separately illustrated in figure A.3.1. Yellow: SU-8, 20 µm height; gray: SU-8, 40 µm height. Scale bars: 500 µm (A), 2 mm (B).
Chip designs for world-to-chip strategy

Fig. A.3.3: CAD chip designs used for chip characterization, e.g. valve function. A. Design applied for total RNA qPCR pre-characterization and chamber-chamber diffusion validation. B. Modification of design shown in (A) bearing changed control valves: two different sizes of valve pads allow for a multiplexed fluid control with one control line only. By varying the induced pressure, either both or only the bigger valve patch can be closed. C. Modified version with dead-end chambers. The volume transferred into these dead-end chambers (black) equals the freshly added probe volume added to the reaction chambers (blue). Gray: fluid layer (AZ-9260, 20 µm height); black: reaction chambers in fluid layer (SU-8, 100 µm height); red: control layer (SU-8, 20 µm height). Scale bars: 1 mm.

Fig. A.3.4: CAD design of the final world-to-chip RT-qPCR chip used for cell-to-chip transfer and (RT)-qPCR experiments. Gray: fluid layer (AZ-9260, 20 µm height); black: reaction chambers in fluid layer (SU-8, 100 µm height); red: control layer (SU-8, 20 µm height). Gray dots in the biggest reaction chambers illustrate columns to prevent the chambers from collapsing. Scale bar: 2 mm.
A.4 Matlab scripts

MATLAB scripts were designed by F. Kurth and S. K. Küster. All programming was conducted by S. K. Küster.

A.4.1 Data evaluation of global calcium increments (chapter 5)

%% Initialization
clear all
close all
fprintf('<strong>SCRIPT STARTED</strong> 
');
fprintf('-------------- 
');
% Start stopwatch timer to measure performance (running time) of the
% complete script.
time_script_start = tic;
% Define path of MATLAB script.
paths.path_script = cd;

%% DOCUMENTATION
% 1. Create a main folder with the name of your experiment
% 2. Create a new folder inside the main folder for the brightfield image
% 3. Copy the brightfield image (tif format, 16 bit) into this folder
% Do not store any other files than this image in this folder
% 4. Create a new folder inside the main folder for the image series
% 5. Copy the image series (tif format, 16 bit, single images) into this
folder
% Do not store any other files than your images in this folder
% 6. Make sure that the files in the image series folder are labeled
correctly
% 7. Run script
% THIS IS VERSION 3 !!!
%% Input Parameters
% directory: insert path directory of files
paths.data_brightfield = char('/directory');
paths.data_img_series = char('/directory');
paths.output = char('/directory');
input_par.plot_ROI_analysis = 0;
input_par.export_to_txt_files = 1;
input_par.export_figures_to_disk = 1;
input_par.workspace_export_to_disk = 1;
input_par.verbose = 'no';

%% Prepare list with files in the brightfield folder
% Create a structure array containing information about all entries (files
% and directories; normal and hidden) as well as '.' and '..' in the data
% folder.
data_folder_info.list_of_folder_content_complete = dir(paths.data_brightfield);
% Remove directories (including . and ..) from list of folder content.
[data_folder_info.list_of_folder_content_no_dirs] =
fun_remove_directories_from_list_of_folder_content(data_folder_info.list_of_folder_content_complete, input_par.verbose);
% Remove hidden files from list of folder content.
[data_folder_info.list_of_folder_content_without_hidden, ~] =
fun_remove_hidden_objects_from_list_of_folder_content(data_folder_info.list_of_folder_content_no_dirs, input_par.verbose);
data_folder_info.list_of_folder_content_brightfield =
data_folder_info.list_of_folder_content_without_hidden;
[no_of_files_in_brightfield_folder, ~] =
size(data_folder_info.list_of_folder_content_brightfield);
if no_of_files_in_brightfield_folder ~= 1
    error('The folder for the brightfield image contains more than one file or
           no file at all.')
end

%% Load brightfield image

file_to_read = data_folder_info.list_of_folder_content_brightfield(1,1).name;
[micrograph, map] = imread(file_to_read,'tif');

% Convert loaded micrograph from uint8 to double format
micrograph_double = double(micrograph);
micrograph_brightfield = micrograph_double;
figure(1),clf
colormap('gray')
handle_image = imagesc(micrograph_brightfield);
colorbar
cb = colorbar('vert');
zlab = get(cb,'ylabel');
set(zlab,'String','Intensity (0-65535)');
title('Brightfield image')
clear micrograph micrograph_double cb zlab file_to_read map

%% Prepare list with files in the image series folder
% Create list of folder content:
% Create a structure array containing information about all entries (files
% and directories; normal and hidden) as well as '.' and '..' in the data
% folder.
data_folder_info.list_of_folder_content_complete =
dir(paths.data_img_series);
% Remove directories (including . and ..) from list of folder content.
[data_folder_info.list_of_folder_content_no_dirs] =
fun_remove_directories_from_list_of_folder_content(data_folder_info.list_of_folder_content_complete, input_par.verbose);
% Remove hidden files from list of folder content.
[data_folder_info.list_of_folder_content_image_series, ~] =
fun_remove_hidden_objects_from_list_of_folder_content(data_folder_info.list_of_folder_content_no_dirs, input_par.verbose);
data_folder_info.list_of_folder_content_image_series =
data_folder_info.list_of_folder_content_without_hidden;
[no_of_files_in_image_series_folder, ~] =
size(data_folder_info.list_of_folder_content_image_series);

%% Loading all images in one 3D matrix

for n = 1:no_of_files_in_image_series_folder
    file_to_read =
data_folder_info.list_of_folder_content_image_series(n,1).name;
    [micrograph, map] = imread(file_to_read,'tif');
    % Convert loaded micrograph from uint8 to double format
micrograph_double = double(micrograph);
% % Pre- Allocation of varibale growing in for-loop
% if n == 1
% micrograph_stack = zeros(size(micrograph,1), size(micrograph,2),
% no_of_img);
% end
% Write current micrograph into the micrograph stack (3D matrix).
micrograph_stack(:,:,n) = micrograph_double;
end
clear n
clear file_to_read micrograph micrograph_double map n

%% Select area for mean background calculation from brightfield image
fprintf('ROI SELECTION FOR BACKGROUND CALCULATION ...
');
% Bring brightfield image in the foreground.
figure(1)
% Select rectangular reagion of interest (ROI)
fprintf('1) Please select ROI for background signal calculation.
');
fprintf('2) Double click on the selection tool to confirm the
');
h = imfreehand;
position = wait(h);
binary_img_mask_BG = createMask(h,handle_image);
clear h handle_image
fprintf('ROI SELECTION FOR BACKGROUND CALCULATION ... COMPLETED.
');
micrograph_BG_select = micrograph_brightfield.*binary_img_mask_BG;
% Replace zero-values by NaN for improved visibility (scaling) in plots.
ind = micrograph_BG_select == 0;
micrograph_BG_select_display = micrograph_BG_select;
micrograph_BG_select_display(ind) = NaN;
clear ind
figure(1),clf
subplot(1,3,1)
colormap('gray')
imagesc(micrograph_brightfield);
colorbar
cb = colorbar('vert');
zlab = get(cb,'ylabel');
set(zlab,'String','Intensity (0-65535)');
title('Brightfield image')
figure(1)
subplot(1,3,2)
colormap('gray')
imagesc(micrograph_BG_select_display);
colorbar
cb = colorbar('vert');
zlab = get(cb,'ylabel');
set(zlab,'String','Intensity (0-65535)');
title('Brightfield image - ROI selection for BG')
clear h position h_im
clear cb zlab

%% Extract average background for the specified ROI from all image series
images
for n = 1: size(micrograph_stack,3)
    micrograph_ROI = micrograph_stack(:,:,n).*binary_img_mask_BG;
    ind = micrograph_ROI > 0;
mean_ROI_BG_series(n,1) = mean2(micrograph_ROI(ind));
end
figure(1)
subplot(1,3,3)
plot(mean_ROI_BG_series)
title('Mean intensity of background ROI over time')
xlabel('Image number')
ylabel('Mean intensity background ROI')

%% Select ROIs for analysis
fprintf('ROI SELECTION FOR CELL ANALYSIS ...
')
% Bring brightfield image in the foreground.
figure(2),clf
colormap('gray')
handle_image = imagesc(micrograph_brightfield);
colorbar
cb = colorbar('vert');
zlab = get(cb,'ylabel');
set(zlab,'String','Intensity (0-65535)');
title('Brightfield image')
% Ask for input to define the total number of ROIs to be evaluated.
no_of_ROIs = input('    How many ROIs to be evaluated? (1-16) [1]: 
');
if isempty(no_of_ROIs)
disp('    No input give. Default setting of 1 file is used.')
    no_of_ROIs = 1;
elseif no_of_ROIs > 16
    disp('    Please select a number between 1 and 16! Default setting of 1 file is used.')
    no_of_ROIs = 1;
elseif no_of_ROIs <= 0
    disp('    Please select a number between 1 and 16! Default setting of 1 file is used.')
    no_of_ROIs = 1;
end
disp(' ')
% Select Roi for each cell
figure(2)
for n = 1:no_of_ROIs
    disp(['    Please select ROI ', num2str(n), ',/', num2str(no_of_ROIs) ' for cell-based signal calculation ...'])
    disp('    Double click on the selected ROI to confirm the selection ...')
    h = imfreehand;
    position = wait(h);
    binary_img_mask_analysis(:,:,n) = createMask(h,handle_image);
    fprintf(['    ROI selection ', num2str(n), ',/', num2str(no_of_ROIs) ' completed.
'])
end
clear n

%% Display an overview of all ROIs and their ROI-number (using the brightfield image)
figure(3),clf
% Plot each ROI in a separate subplot.
for n = 1:size(binary_img_mask_analysis,3)
    % Adjust the subplot to the number of defined ROIs.
    if no_of_ROIs == 1

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subplot(1,1,n)

elsif no_of_ROIs > 1 && no_of_ROIs <= 2
    subplot(1,2,n)
elsif no_of_ROIs > 2 && no_of_ROIs <= 4
    subplot(2,2,n)
elsif no_of_ROIs > 2 && no_of_ROIs <= 6
    subplot(2,3,n)
elsif no_of_ROIs > 6 && no_of_ROIs <= 9
    subplot(3,3,n)
elsif no_of_ROIs > 9 && no_of_ROIs <= 12
    subplot(3,4,n)
elsif no_of_ROIs > 12 && no_of_ROIs <= 16
    subplot(4,4,n)
end

% Prepare image for plotting.
current_image_for_plotting =
    micrograph_brightfield.*binary_img_mask_analysis(:,:,n);
% Replace zero-values by NaN for improved visibility (scaling) in plots.
ind = current_image_for_plotting == 0;
current_image_for_plotting(ind) = NaN;
colormap('gray')
handle_image = imagesc(current_image_for_plotting);
colorbar
    cb = colorbar('vert');
    zlab = get(cb,'ylabel');
    set(zlab,'String','Intensity (0-65535)');
title(['ROI ', num2str(n)])
end
clear n current_image_for_plotting ind
fprintf('ROI SELECTION FOR CELL ANALYSIS ... COMPLETED.\n\n')

%% Extract individual intensity from each ROI from each image
disp('ROI ANALYSIS ...')
binary_img_mask_analysis = double(binary_img_mask_analysis);
for n = 1:no_of_ROIs % cycle through ROIs
disp(['    Analysis of ROI ', num2str(n), '/', num2str(no_of_ROIs)])
    for m = 1:no_of_files_in_image_series_folder % cyle through images

        if input_par.plot_ROI_analysis == 1;
            figure(4),clf
            subplot(1,2,1)
            colormap('gray')
            imagesc(micrograph_stack(:,:,m))
            colorbar
            cb = colorbar('vert');
            zlab = get(cb,'ylabel');
            set(zlab,'String','Intensity (0-65535)');
            title('Current image from imgage series')
        end

    end

% Create the mean intensity value of the ROI
    cropped_image =
    micrograph_stack(:,:,m).*binary_img_mask_analysis(:,:,n);
    ind = cropped_image > 0;
    mean_ROI_cells(m,n) = mean2(cropped_image(ind));
% Replace zero-values by NaN for improved visibility (scaling) in plots.
    ind = cropped_image == 0;
cropped_image_display = cropped_image;
cropped_image_display(ind) = NaN;
clear ind 
if input_par.plot_ROI_analysis == 1;
    subplot(1,2,2)
    colormap('gray')
    imagesc(cropped_image_display)
    colorbar
    cb = colorbar('vert');
    zlab = get(cb,'ylabel');
    set(zlab,'String','Intensity (0-65535)');
    title('Current image from image series with current ROI')
    pause(0.25)
end 
end
clear m n cb zlab ind
%%
figure(5), clf
subplot(1,2,1)
hold on
handle_1 = plot(mean_ROI_cells);
handle_2 = plot(mean_ROI_BG_series, 'LineStyle', '--', 'LineWidth', 0.5,
'Color', 'k');
hold off
title('Mean intensity as a function of image number')
xlabel('Image number')
ylabel('Intensity')
for n = 1:size(mean_ROI_cells,2)
    set(handle_1(n),'DisplayName', ['ROI ',num2str(n)])
end
clear n
set(handle_2(1),'DisplayName', 'BG')
legend('show')
% Calculate the background-corrected mean values for each ROI.
for n = 1:size(mean_ROI_cells,2)
    mean_ROI_cells_BG_corrected(:,n) = mean_ROI_cells(:,n) - mean_ROI_BG_series;
end
clear n
subplot(1,2,2)
handle_3 = plot(mean_ROI_cells_BG_corrected);
title('With background correction')
xlabel('Image number')
ylabel('Intensity')
for n = 1:size(mean_ROI_cells_BG_corrected,2)
    set(handle_3(n),'DisplayName', ['ROI ',num2str(n), ' BG corr.'])
end
clear n
clear handle_1 handle_2 handle_3
fprintf('ROI ANALYSIS ... COMPLETED.')
legend('show')

% Exporting results to text files
if input_par.export_to_txt_files == 1
    fprintf('Exporting results to text files ... \n');
cd(paths.output)
dlmwrite('mean_ROI_BG_series.txt', mean_ROI_BG_series,'delimiter','\t');
dlmwrite('mean_ROI_cells.txt', mean_ROI_cells, 'delimiter','\t');
dlmwrite('mean_ROI_cells_BG_corrected.txt', mean_ROI_cells_BG_corrected, 'delimiter','\t');

if input_par.export_figures_to_disk == 1
    fprintf('Exporting figures to disk ... \n');
    set(0, 'ShowHiddenHandles', 'on');
    list_of_figures = get(0, 'Children');
    % Change to evaluation directory for export of figure(s)
    cd(paths.output)
    current_path = cd;
    fprintf([' ', 'Exporting figure(s) to ', current_path, ' \n'])
    % Cycle through choosen figures for export.
    for n = 1:size(list_of_figures,1)
        % Bring figure to front for export.
        figure(list_of_figures(n))
        % Create filename for figure export.
        filename = ['figure_', num2str(list_of_figures(n))];
        % Export selected figures as png-file with fixed size.
        set(gcf, 'PaperPositionMode', 'manual');
        set(gcf, 'PaperUnits', 'inches');
        set(gcf, 'PaperPosition', [2 1 16 9]);
        extension = '.png';
        helpvar = ['print -dpng -r300 ', filename, extension];
        eval(helpvar)
        % Save selected figures as fig-file.
        extension = '.fig';
        filename = [filename, extension];
        eval(['saveas(list_of_figures(n), ', filename ')]);
    end
    clear n
    % Change back to the script directory.
    cd(paths.path_script)
    clear n filename extension helpvar list_of_figures current_path
    fprintf('Exporting figures to disk ... completed. \n');
else
    fprintf('Exporting figures to disk ... SKIPPED. \n');
end

% Export the whole workspace to a file
if input_par.workspace_export_to_disk == 1
    fprintf('Exporting MATLAB workspace to disk ... \n');
    % Change to evaluation directory for export of figure(s)
    cd(paths.output)
    save('full_workspace.mat')
    % Change back to the script directory.
    cd(paths.path_script)
    fprintf('Exporting MATLAB workspace to disk ... completed. \n');
else
    fprintf('Exporting MATLAB workspace to disk ... SKIPPED. \n');
end
end

%% Lead Out

cd(paths.path_script)
total_script_run_time = toc(time_script_start);
% Stop stopwatch timer to measure the execution time of the whole script.
fprintf('--------------
');
fprintf('<strong>SCRIPT FINISHED</strong>
')
fprintf(['(Total run time: ', num2str(total_script_run_time), ' sec = ',
        num2str(floor(total_script_run_time/60)), ' min and ',
        num2str(rem(total_script_run_time,60)), ' sec)\n'])
clearvars time_script_start total_script_run_time
A.4.2 Data evaluation of calcium sparks (initial test phase)

%% Initialization
clc
clear all
close all

%% DOCUMENTATION
% 1. Create a folder with the name of your experiment
% 2. Create a folder inside this folder named images
% 3. Copy all your images (tif) into this folder
% 4. Do not store any other files than your images in this folder
% 5. Set the path under 'Input Parameters' to the folder containing the images
% 6. Run script
% 7. Check 'MATLAB evaluation' folder
% THIS IS VERSION 3 !!!

%% Input Parameters
path_data = char('/directory'); % directory: insert path directory of files
intensity_ratio_threshold = 1.22;
hist_analysis = 1;
binning_width = 0.0001;
detailed_plotting = 1;
export_figures = 1;
summary_output = 1;

path_script = cd;
filenames = dir(path_data);
[no_of_img, ~] = size(filenames);
no_of_img = no_of_img - 2; % take care to set this back to "no_of_img - 2"

for n = 1+2:no_of_img+2
    file_to_read = filenames(n,1).name;
    [~,micrograph, map] = imread(file_to_read,'png');
    [~,micrograph, map] = imread(file_to_read,'tif');
    micrograph_double = double(micrograph);
    if n == 1
        micrograph_stack = zeros(size(micrograph,1), size(micrograph,2), no_of_img);
    end
    micrograph_stack(:,:,n-2) = micrograph_double;
end

%% Creating an "envelope-image"
envelope_img = max(micrograph_stack,[],3);
figure(1),clf
colormap('jet')
imagesc(envelope_img)
colorbar
cb = colorbar('vert');
Appendix

```matlab
zlab = get(cb,'ylabel');
set(zlab,'String','Intensity (0-65535)');
title('envelope-image (scaled)')

%% Creating a "mean-image"
mean_image = sum(micrograph_stack,3)./size(micrograph_stack,3);
figure(2),clf
colormap('jet')
imagesc(mean_image)
colorbar
cb = colorbar('vert');
zlab = get(cb,'ylabel');
set(zlab,'String','Intensity (0-65535)');
title('mean-image (scaled)')

%% Individual image spark detection
summary = [NaN NaN NaN NaN NaN NaN NaN NaN];
% Cycle through each individual micrograph in the micrograph_stack.
for n = 1:size(micrograph_stack,3)
    % Extract current micrograph from the stack containing all micrographs.
    current_micrograph = micrograph_stack(:,:,n);
    % Calculate the intensity ratio of the current micrograph to the
    % "mean-image" for each pixel.
    current_to_mean_ratio = current_micrograph./mean_image;
    % Test which pixels have an intensity ratio which exceeds the threshold
    % value.
    index_over_threshold_stack = current_to_mean_ratio > intensity_ratio_threshold;
    % Count the number of pixels with intensity ratios which exceed the
    % threshold value.
    n_of_pixels_above_threshold = sum(sum(index_over_threshold_stack,1),2);
    % Rename variable
    binary_image = index_over_threshold_stack;
    % Connect positive pixels which are diagonally bridged.
    % ("Uses diagonal fill to eliminate 8-connectivity of the background.")
    binary_image = bwmorph(binary_image,'diag');
    % Detect each element in the binary image.
    bwconncomp_results = bwconncomp(binary_image);
    % Extract number of detected elements.
    n_detected_elements = size(bwconncomp_results.PixelIdxList,2);
    % For each detected element, find the maximum intensity, the position
    % in the image and the intensity ratio.
    for m = 1:n_detected_elements
        % Extract the list of pixels for the current element.
        current_element = bwconncomp_results.PixelIdxList{m};
        % Find out which element in the pixel list has the highest
        % intensity and determine the position of this element in the pixel
        % list.
        [max_int, ind_max_int] = max(current_micrograph(current_element));
        % Extract the linear index of the pixel with the highest intensity
        % for the current element from the pixel list.
        lin_ind = bwconncomp_results.PixelIdxList{m}(ind_max_int);
        % Use the linear index to extract the intensity ratio of the
        % current micrograph to the "mean-image" for the most intense pixel
        % in the current element.
        current_to_mean_ratio_AAABBB = current_to_mean_ratio(lin_ind);
        % Determine the subscripts equivalent to the linear index.
```
current_size = size(binary_image);
[I,J] = ind2sub(current_size,lin_ind);
summary_current_element(1,1) = n;
summary_current_element(1,2) = n_detected_elements;
summary_current_element(1,3) = m;
summary_current_element(1,4) = size(current_element,1);
summary_current_element(1,5) = max_int;
summary_current_element(1,6) = current_to_mean_ratio_AAABBB;
summary_current_element(1,7) = J;
summary_current_element(1,8) = I;
summary = [summary; summary_current_element];
% Delete initialization values after the first row of real values
% has been added.
if size(summary,1) == 2 & isnan(summary(1,1)) == 1
    summary(1,:) = [];
end
end

cleaned_micrograph = current_micrograph.*index_over_threshold_stack;

%figure(99),clf
%imagesc(cleaned_micrograph)

%%STATS = regionprops(cc, 'MaxIntensity')
%result = input('Press enter for next image.);
end

% Intensity ratio of envelope-image to mean-image
env_mean_ratio = envelope_img./mean_image;
figure(3),clf
colormap('jet')
imagesc(env_mean_ratio)
colorbar
title('Intensity ratio of envelope-image to mean-image')

% Calculating the number of pixels above the intensity ratio threshold
index_over_threshold = env_mean_ratio > intensity_ratio_threshold;
number_of_pixels_above_threshold = sum(sum(index_over_threshold));
% Isolating all values in the intensity ratio of envelope-image to
% mean-image matrix which surpass the threshold.
env_mean_ratio_threshold = env_mean_ratio.*index_over_threshold;
figure(4),clf
colormap(jet(ceil(max(max(env_mean_ratio_threshold))) -
floor(min(min(env_mean_ratio_threshold)))))
imagesc(env_mean_ratio_threshold)
colorbar
title(['Intensity ratio of envelope-image to mean-image, threshold analysis
(threshold =', num2str(intensity_ratio_threshold), ', number of pixels above
threshold =', num2str(number_of_pixels_above_threshold)])

%% Histogram analysis
if hist_analysis == 1
    figure(5),clf
    index = env_mean_ratio_threshold == 0;
    env_mean_ratio_threshold(index) = NaN;
    % Reshape matrix into column vector
    env_mean_ratio_threshold_hist = reshape(env_mean_ratio_threshold,numel(env_mean_ratio_threshold),1);
    % Binning definition
    binning = [min(env_mean_ratio_threshold_hist);binning_width:max(env_mean_ratio_thres
hold_hist);

% Plot histogram
subplot(1,2,1)
hist(env_mean_ratio_threshold_hist,binning)
title(['Histogram analysis of all intensity ratios (envelope/mean) above 
threshold (threshold = ', num2str(intensity_ratio_threshold), ') 
number of 
pixels above threshold = ', num2str(number_of_pixels_above_threshold), ') 
binning width = ', num2str(binning_width))
xlabel('intensity ratio envelope-image over mean-image')
ylabel('frequency')
h = findobj(gca,'Type','patch');
set(h,'FaceColor','b','EdgeColor','b')
xlim([min(binning),max(binning)])

subplot(1,2,2)
hist(env_mean_ratio_threshold_hist,binning)
title(['Histogram analysis of all intensity ratios (envelope/mean) above 
threshold (threshold = ', num2str(intensity_ratio_threshold), ') 
number of 
pixels above threshold = ', num2str(number_of_pixels_above_threshold), ') 
binning width = ', num2str(binning_width))
xlabel('intensity ratio envelope-image over mean-image')
ylabel('frequency')
h = findobj(gca,'Type','patch');
set(h,'FaceColor','b','EdgeColor','b')
axis([min(binning),max(binning),0,5])

end

%% Find position of each pixel above threshold
[row, col] = find(index_over_threshold);

%% Calculate the intensity ratio of the current image to the previous image
for n = 1:size(micrograph_stack,3)-1
    ratio_to_previous(:,:,n) =
    micrograph_stack(:,:,n+1)./micrograph_stack(:,:,n);
end

%% Calculate the intensity ratio of the current image to the first image
for n = 1:size(micrograph_stack,3)
    ratio_to_first(:,:,n) =
    micrograph_stack(:,:,n)./micrograph_stack(:,:,1);
end

%% Calculate the intensity ratio of the current image to a set of previous 
images
for n = 10:size(micrograph_stack,3)-1
    ratio_to_set_of_previous(:,:,n) =
    micrograph_stack(:,:,n+1)./mean(micrograph_stack(:,:,n-7:n-2),3);
end

%% Show the intensity over time of one pixel and all surrounding pixels
if detailed_plotting == 1
    for n = 1:size(row,1)
        figure(200+n), clf
        subplot(1,2,1)
        if (row(n) ~= 1) && (row(n) ~= size(micrograph, 1)) && (col(n) ~= 1) &&
            (col(n) ~= size(micrograph, 2))
            hold on
            intensity_over_time = squeeze(micrograph_stack(row(n),col(n),:));
            plot(intensity_over_time,'-k')
            intensity_over_time = squeeze(micrograph_stack(row(n)-1,col(n),:));
            % Plot histogram
            subplot(1,2,1)
            hist(env_mean_ratio_threshold_hist,binning)
            title(['Histogram analysis of all intensity ratios (envelope/mean) above 
threshold (threshold = ', num2str(intensity_ratio_threshold), ') 
number of 
pixels above threshold = ', num2str(number_of_pixels_above_threshold), ') 
binning width = ', num2str(binning_width))
            xlabel('intensity ratio envelope-image over mean-image')
            ylabel('frequency')
            h = findobj(gca,'Type','patch');
            set(h,'FaceColor','b','EdgeColor','b')
            xlim([min(binning),max(binning)])
            subplot(1,2,2)
            hist(env_mean_ratio_threshold_hist,binning)
            title(['Histogram analysis of all intensity ratios (envelope/mean) above 
threshold (threshold = ', num2str(intensity_ratio_threshold), ') 
number of 
pixels above threshold = ', num2str(number_of_pixels_above_threshold), ') 
binning width = ', num2str(binning_width))
            xlabel('intensity ratio envelope-image over mean-image')
            ylabel('frequency')
            h = findobj(gca,'Type','patch');
            set(h,'FaceColor','b','EdgeColor','b')
            axis([min(binning),max(binning),0,5])
end
Appendix

```matlab
plot(intensity_over_time,'-r')
intensity_over_time = squeeze(micrograph_stack(row(n)-1,col(n)+1,:));
plot(intensity_over_time,'-r')
intensity_over_time = squeeze(micrograph_stack(row(n),col(n)+1,:));
plot(intensity_over_time,'-m')
intensity_over_time = squeeze(micrograph_stack(row(n)+1,col(n)+1,:));
plot(intensity_over_time,'-m')
intensity_over_time = squeeze(micrograph_stack(row(n)+1,col(n),:));
plot(intensity_over_time,'-g')
intensity_over_time = squeeze(micrograph_stack(row(n)+1,col(n)-1,:));
plot(intensity_over_time,'-g')
intensity_over_time = squeeze(micrograph_stack(row(n),col(n)-1,:));
plot(intensity_over_time,'-b')
intensity_over_time = squeeze(micrograph_stack(row(n)+1,col(n)-1,:));
plot(intensity_over_time,'-b')
hold off

title(['Intensity over time; pixel: x=', num2str(col(n)), ' y=', num2str(row(n))])
xlabel('image number')
ylabel('absolute intensity')
legend('center', 'top', 'topright', 'right', 'bottomright', 'bottom', 'bottomleft', 'left', 'topleft')

subplot(1,2,2)
hold on
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n),col(n),:));
plot(ratio_to_set_of_previous_over_time,'-k')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n)-1,col(n),:));
plot(ratio_to_set_of_previous_over_time,'-r')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n)-1,col(n)+1,:));
plot(ratio_to_set_of_previous_over_time,'-r')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n),col(n)+1,:));
plot(ratio_to_set_of_previous_over_time,'-m')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n)+1,col(n)+1,:));
plot(ratio_to_set_of_previous_over_time,'-m')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n)+1,col(n),:));
plot(ratio_to_set_of_previous_over_time,'-g')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n)+1,col(n)-1,:));
plot(ratio_to_set_of_previous_over_time,'-g')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n),col(n)-1,:));
plot(ratio_to_set_of_previous_over_time,'-b')
ratio_to_set_of_previous_over_time = squeeze(ratio_to_set_of_previous(row(n)+1,col(n)-1,:));
plot(ratio_to_set_of_previous_over_time,'-b')
line([1 no_of_img], [intensity_ratio_threshold intensity_ratio_threshold], 'LineStyle', ':')
```

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hold off
title(['Ratio to mean intensity value of a set of previous images
over time; pixel: x=', num2str(col(n)), ' y=', num2str(row(n))])
xlabel('image number')
ylabel('ratio')
legend('center', 'top', 'topright', 'right', 'bottomright',
'bottom', 'bottomleft', 'left', 'topleft')
else
fprintf('"Hot" pixel at the very rim of the image - evaluation
skipped 

');
end
end
end

%% Write summary file
if summary_output == 1
cd..
if exist('MATLAB evaluation','dir') ~= 7
mkdir('MATLAB evaluation');
end
cd('MATLAB evaluation')
file_1 = fopen('summary.txt','w');
fprintf(file_1,'MATLAB spark detection summary file (Version 3)

');
fprintf(file_1,'Evaluation performed on ', date, ' at ', datestr(now,
'HH:MM:SS'), '

');
fprintf(file_1,'Path = ', path_data, '

');
fprintf(file_1,'Threshold = ', num2str(intensity_ratio_threshold)

');
fprintf(file_1,'Binning width (histogram) = ', num2str(binning_width)

');
fprintf(file_1,')[Note: Top left corner of image is (1,1)]

');
fprintf(file_1,'][Frame // Total Number of Elements // Number of Element //
Number of Pixels // Max. Abs. Intensity // I/I(mean) // x // y]

');
% for n = 1:size(col,1)
% fprintf(file_1,['(', num2str(col(n)), ',', num2str(row(n)), ')\n']);
% end
% for n = 1:size(summary,1)
% fprintf(file_1,summary(n,:));
% end
%fprintf(file_1,['Path = ' path_data '\n\n'])
close(file_1);
dlmwrite('summary.txt', summary, '-append', 'delimiter', '	')
cd(path_data)
end

%% Figure Export (as . fig and/or image files)
if export_figures == 1
set(0,'ShowHiddenHandles', 'on')
list_of_figures = get(0, 'Children');
% Change to evaluation directory for export of figure(s)
cd(path_data)
cd..
cd('MATLAB evaluation')
current_path = cd;
fprintf({'Exporting figure(s) to ', current_path, '\n\n'})
% Cycle through choosen figures for export.
for n = 1:size(list_of_figures,1)
% Bring figure to front for export.
figure(list_of_figures(n))
% Create filename for figure export.
filename = {'figure_','num2str(list_of_figures(n))'};
% Export selected figures as png-file with fixed size.
set(gcf, 'PaperPositionMode', 'manual');
set(gcf, 'PaperUnits', 'inches');
set(gcf, 'PaperPosition', [2 1 16 9]);
extension = '.png';
s = {'print -dpng -r300 ', filename, extension};
eval(s)
% Save selected figures as fig-file.
extension = '.fig';
filename = [filename, extension];
eval(['saveas(list_of_figures(n), ','filename')])
end
% Change back to the data directory.
cd(path_data);
end

%% Lead Out
cd(path_script)
disp('Script finished.')
Curriculum vitae
Curriculum vitae

**Personal data**

Name: Felix Kurth  
Born: January 19, 1982 in Haan, Germany  
Nationality: German

**Education**

2010 - 2015  PhD student, Bioanalytics Group, Department of Chemistry and Applied Biosciences, ETH Zürich, Switzerland  
Supervisors: Prof. Dr. P.S. Dittrich, Prof. Dr. R. Müller, and Dr. A. Franco-Obregón

2009 - 2010  Diploma thesis, Laboratory of Chemical Biotechnology, TU Dortmund, Germany  
Supervisors: Prof. Dr. A. Schmid, Dr. L.M. Blank, and Dr. B. Ebert

2006  Studies at the Royal Technical University of Stockholm (KTH), Sweden

2002 - 2010  Studies in Bioengineering, TU Dortmund, Germany

1988 - 2001  Obligatory education in Germany, graduation at grammar school

**Further curricular activities, work experience**

2014  Entrepreneurship project “venture challenge”, venturelab, ETH Zurich, Switzerland

2011 - 2013  Supervision of visiting scientist

2010 - 2011  Teaching assistant in the practical lab course on Biological Chemistry at ETH Zürich, Switzerland (Prof. Dr. P. Kast, Laboratory of Organic Chemistry, ETH Zürich, Switzerland)

2008 - 2009  Industrial placement at Lonza, Visp, Switzerland

2004 - 2008  Student assistant at the Laboratory of Fluid Separations, TU Dortmund, Germany (Prof. Dr. A. Górak)

2003  Industrial placement at company Rasspe, Solingen, Germany

2001 - 2002  Civilian service, Arbeiterwohlfahrt, Solingen, Germany

**Grants**

2013  2013 SCNAT/SCS Chemistry Travel Award, Mechano-Actuated Ca\(^{2+}\) Transit on a Microfluidic Device Reveals Spatial and Temporal Characteristics. International Conference on Microtechnologies in Medicine and Biology MMB2013, 2013, April 10-12, Marina del Rey, California, USA

2013  GdCH Travel Grant, A Microfluidic Platform for the Online Monitoring of Mechano-Activated Intracellular Ca\(^{2+}\) Signaling. ANAKON 2013, 2013, March 4-7, Essen, Germany
Scientific articles


Further peer reviewed publications


A.J. Trüssel, D.J. Webster, N. Curq, **F. Kurth**, P.S. Dittrich, R. Müller, Mechanical systems biology in bone: Towards high-throughput quantification of gene expression in individual osteocytes. *2012*, J. Biomech., 45 (S1), 433

Oral presentations

**F. Kurth**, R.E. Wilson, A.J. Trüssel, D.J. Webster, R. Müller, P.S. Dittrich, A world-to-chip strategy for the analysis of in vivo stimulated cells. Meeting of Swiss Analytical Scientists (CHanalysis 2013), 2013, November 29-30, Beatenberg, Switzerland


**Poster presentations**


F. Kurth, R.E. Wilson, A.J. Trüssel, D.J. Webster, R. Müller, P.S. Dittrich, Towards microfluidic analysis of *in vivo* stimulated bone cells. 6th Symposium SSCI (Scholarship Fund of the Swiss Chemical Industry), 2013, December 17, Zürich, Switzerland

S. Stratz, K. Eyer, F. Kurth, P.S. Dittrich, Quantitative analysis of single *E. coli* lysates. 6th Symposium SSCI (Scholarship Fund of the Swiss Chemical Industry), 2013, December 17, Zürich, Switzerland


S. Stratz, K. Eyer, F. Kurth, P.S. Dittrich, Towards quantitative analysis of *E. coli* lysates. International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS 2013), 2013, October 27-31, Freiburg, Germany

