Microwell devices for single-cell analyses

Sara Lindström

Royal Institute of Technology
School of Biotechnology

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Sara Lindström was born in Piteå 1980. Completed Stockholm Graduate School of Molecular Life Sciences at the Karolinska Institute (KI) in 2004. Received her Master of Science in Molecular Biotechnology at the Royal Institute of Technology (KTH) in 2005. Started her doctoral studies at the Division of Nanobiotechnology, Royal Institute of Technology in 2006.
Abstract.

Powerful tools for detailed cellular studies are emerging, increasing the knowledge of the ultimate target of all drugs: the living cell. Today, cells are commonly analyzed in ensembles, i.e., thousands of cells per sample, yielding results on the average response of the cells. However, cellular heterogeneity implies the importance of studying how individual cells respond, one by one, in order to learn more about drug targeting and cellular behavior. In vitro assays offering low volume sampling and rapid analysis in a high-throughput manner are of great interest in a wide range of single-cell applications.

This work presents a microwell device in silicon and glass, developed using standard microfabrication techniques. The chip was designed to allow flow-cytometric cell sorting, a controlled way of analyzing and sorting individual cells for dynamic culture and clone formation, previously shown in larger multiwell plates only. Dependent on the application, minor modifications to the original device were made resulting in a group of microwell devices suitable for various applications. Leukemic cancer cells were analyzed with regard to their clonogenic properties and a method for investigation of drug response of critical importance to predict long-term clinical outcome, is presented. Stem cells from human and mouse were maintained pluripotent in a screening assay, also shown useful in studies on neural differentiation.

For integrated liquid handling, a fluidic system was integrated onto the chip for directed and controlled addition of reagents in various cell-based assays. The chip was produced in a slide format and used as an imaging tool for low-volume sampling with the ability to run many samples in parallel, demonstrated in a protein-binding assay for a novel bispecific affinity protein. Moving from cells and proteins into genetic analysis, a method for screening genes from clones in a rapid manner was shown by gene amplification and mutation analysis in individual wells. In summary, a microwell device with associated methods were developed and applied in a range of biological investigations, particularly interesting from a cell-heterogeneity perspective.

Keywords: microwell, miniaturization, microfluidics, cell culture, single-cell, clone, imaging, stem cell, cancer, low volume, high-throughput. © Sara Lindström 2009
“Science is organized knowledge. Wisdom is organized life.”

-Immanuel Kant
To Jimmy, 

for supporting me in doing what I like the most.
LIST OF PUBLICATIONS

This thesis is based upon the following publications, which are referred to in the text by their Roman numerals (I-V). The five original papers are found in the appendix.


*Authors have contributed equally to the work.

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Prologue.

Today, many large and bulky things are transferred into smaller, more convenient formats. Size reduction has become a natural part in our everyday life, covering a spectrum of useful objects ranging from electronic equipment such as mobile phones and computers to outdoor gear such as life jackets and mats for comfortable sleeping during hiking holidays. As the surrounding world goes smaller and smaller, performance demands are continuously increasing. Products tend to adapt to “all-in-one” concepts, e.g. mobile phones with integrated mp3-players, emails, cameras etc. and customers are increasingly appreciating compact formats, quality and usefulness. With the technological development, new markets are being formed and consumers buy products they did not even know they needed (or that the product even existed) a couple of months earlier.

With ancestry in the semiconductor and microelectronic industry, the field of micro-and nanotechnologies is continuously expanding into new areas, for example life sciences and molecular biology. To learn more about diseases, tools for investigating cellular processes in the body (in vivo), or outside of the body (in vitro) are needed. Animal- and cell-models are frequently applied systems and in drug development, accepted data from these systems is required by regulatory authorities before a compound can be transferred into clinical phase, e.g. efficacy-testing in humans. A cell is the smallest living unit of an organism and thus the ultimate target for all drugs. Proteins and DNA are crucial parts of the machinery maintaining cellular functions. When a cell in the human body (e.g. muscle, brain or blood cell) somehow fails, the downstream effects depend on the particular role of that cell and can thus result in everything from flaking after sun bathing to life-threatening diseases like cancer. Cell-based methods are commonly used in drug screening where a range of compounds and combinations thereof are to be tested on multiple cell samples. The testing of as many compounds as possible against a given amount of patient sample requires a tool where low volume sampling is sufficient for accurate results and on which a high number of experiments per cm² can be performed. This thesis describes the development and applications of miniaturized tools for cell culture and other types of analyses commonly used in molecular biotechnology. The novelty lies in the small format, the high throughput and above all: the applications and possibilities enabled by the tools.
2. Microwell devices for single-cell analyses
INTRODUCTION
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1st chapter: Miniaturization.

There are many reasons why scaling down in size is beneficial for analyses in molecular biology. First, the molecules of interest are small (nm) implying that micrometer-sized tools for analysis should be better suited than conventional laboratory beakers and test tubes (cm). Second, the sometime small amount of biological sample enables more experiments to be run, leading to better result interpretation. Third, by using smaller volumes of expensive reagents, economical savings can be made. Further advantages are faster analysis, space for parallelization, automation and integration of sequential analyses, portability and waste reduction. The manufacture and utilization of microsystems have, during the past 20 years, practically exploded and given rise to a community of research driven by the belief that downscaling will advance the knowledge and obtainable achievements in the field of life science because of the advantages mentioned, as well as in many other areas.

The papers presented and discussed in this thesis deal with the development and applications of a miniaturized microwell plate. The general idea of developing such a plate was to make use of the above stated benefits of miniaturization and employ them for cellular research. Figure 1 shows an illustration of a microwell chip,
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providing many experiments in small wells to be run in parallel, for example useful in live cell imaging using standard microscopy. This chapter describes an overview of the field of miniaturization (from a life science point of view) and glances upon the fabrication processes involved in the production of the microwell plate.

History and trends.

30 years ago the first miniaturized analytical tool, a silicon-made gas chromatographic analyzer (Terry et al., 1979) was presented. The total channel length of the device was 1.5 m and though shown capable of separating components of a sample mixture in a few seconds, there were practically no response from the scientific community. During the following 10 years, a lot of work on miniaturization of devices in silicon focused on the fabrication of components such as micropumps, microvalves and sensors, as reviewed by Reyes and colleagues (Reyes et al., 2002). In parallel, analytical chemistry and methods thereof became a promising area for miniaturized devices. Today, the buzzword “nano” is part of practically every other scientific grant application and a greater response to the community is given. The field of microfabrication originated in the semi-conductor and microelectronic industry and commonly used synonyms are microelectromechanical systems (MEMS), microsystem technology, and micromachines. In 1990, the term “Micro Total Analysis Systems” (μTAS) was introduced (Manz, 1990) describing a microsystem carrying out an entire analytical process such as sampling, sample transport, chemical reactions and/or separations, purification and detection on a single chip. The field has grown exponentially and gained attraction since then, and is nowadays synonymously called Lab-on-a-Chip (Dittrich and Manz, 2006) which is also the name of a journal in the field (LOC). Briefly, the vision behind the LOC concept is to shrink an entire laboratory workflow with complete instrumentation into a small chip, with for example a footprint of 1 x 1 cm². Manz and co-workers are continuously updating on the latest achievements in the field in a series of reviews (Auroux et al., 2002; Dittrich et al., 2006; Reyes et al., 2002; Vilkner et al., 2004; West et al., 2008) in addition to topical reviews by other groups (Mijatovic et al., 2005; Sato et al., 2009; Whitesides, 2006).

The majority of the research within the field of miniaturization has been directed toward the biological and life sciences. Bio-MEMS, biochips, cell/protein/DNA arrays, microfluidics, microsystems, and medical MEMS are
additional frequently used terms. Generally, the 1st generation of chips focused on analytical chemistry, the 2nd generation on chemical synthesis, and more recently the 3rd generation on biology. Applications ranging from nucleic acids analysis (Landers, 2003; Zhang et al., 2006) to protein applications (Lion et al., 2004; Wang, 2002) and cell-based assays (Dufva, 2009; Toner and Irimia, 2005; West et al., 2008) have been developed side by side, with shifting trends and areas of focus over the years. In the early days genomics on chip was an active area of research, though Ewing et al. presented early work on single cells in the proceedings of the μTAS conference ’96’. Nowadays, the interest in utilizing chips for the study of cells and tissue is enormous, and a continuously growing segment is the analysis of single cells, upon which this thesis focuses.

There are two main drives that motivates researchers in the field, namely i) technology with increasingly challenging structures and functionalities, or ii) certain applications where tools for increased knowledge and practical use is the aim. A natural trend in the field is the massive push for further exploring the second alternative. We have the tools, and the next step is to put them to use and steer the development to where they are most beneficial. Many microsystems have shown improved performance compared to conventional techniques and novel studies can be made possible at the microscale due to other physical phenomena than we are used to in our everyday experience. For example, at the microscale flows are purely laminar and diffusion is the basic mechanism for mixing, since turbulence is very seldom encountered. High surface-to-volume ratios, evaporation, liquid handling, surface tension, bubble formation are other phenomena behaving in a non-intuitive manner at the microscale.

It is important for application-driven chip-based technologies to reach the end-users and face the relevant questions that appear in for example a clinical lab. Of uttermost importance for the field, and also what has led the community to its current position, is the cross-disciplinary thinking and acting: bridging technology and medicine. Historically it might appear as if the development of microfluidic devices aimed at sophistication through integration of many experimental steps. But for a device to be user-friendly and generally adopted it must be robust and easy to use. Sometimes the simplest structure (technologically- and fabrication-wise) can be the best structure for a certain purpose. For example, a straight channel or a microwell can be very useful for studying live cell-cell interactions by microscopy. The value lies
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primarily in the small size of the channel or well, maintaining the object of interest in the field of view for analysis. Spotting the potential use of a scientific idea and adapting it for commercialization by improving user-friendliness might primarily not be the responsibility of the academic researcher, but rather in the hands of industrial partners or investors. Nevertheless it helps a great deal if the potential of the device is considered immediately at the outset (i.e. often in an academic setting), for commercialization as well as user-friendliness and robustness of the device. Also, the chip-to-world interface, i.e. final use and connections of the device to standard laboratory equipment, need consideration during the phases of development. Instruments will (most probably) not change according to a chip, but the chip can easily be designed to fit into, or be integrated with, conventional laboratory equipment in a user-friendly manner. One of many exceptions for the need of instrument compatibility is a portable device where the key asset is the mobility.

The awareness of miniaturized tools in clinical and biological settings is ever (yet slowly) increasing. Biologically relevant publications demonstrating the potential, the use, and the obtained benefits of miniaturized systems in high-impact biological journals are good ways of advertising the field of miniaturization, thus reaching out to the envisioned end-users. Still, the “killer-application” for each target group must be demonstrated to offer robustness and improvements over currently available technology before an average non-technological biological laboratory will even consider using microdevices in their own research. Until then the standard tools, which these researchers are used to and trust in, often continue to be their method of choice until proven the value of the miniaturized device. Many manufacturing companies provide help with design and fabrication of microdevices, but biologists’ seldom (if ever) contact these directly wherefore a strong link between the biologists and the micro- and nanogroups or manufacturer companies is needed. Groups with cross-disciplinary knowledge, or fruitful collaborations between “micro/nano-“ and “bio”-groups, will hopefully succeed in reaching the end users for discoveries previously not possible. Today, some companies have “ready-to-use” chips on the market providing application-specific miniaturized assays (Roche; Phadia; Agilent; Gyros) and with increasing interest in miniaturization from leading life science businesses, the trend for commercialization of useful structures is probably still in its infancy.
Microfabrication.

New methods of fabrication and materials are constantly being introduced to the field. Silicon and glass were the materials first explored as they were well known from the semi-conductor industry (Manz et al., 1992; van den Berg and Lammerink, 1997). For some applications though, transparency and cheaper fabrication was crucial which led to the increased attention to polymers and soft lithography for microfabrication (Ng et al., 2002; Whitesides and Stroock, 2001). Being able to produce microstructures rapidly and inexpensively in a standard molecular biology laboratory is exceedingly attractive and is realized by polymer fabrication. Thus, the possibility of making intelligent choices of what material to use and how to combine them for a specific target application is today in our hands.

Processes involved in the fabrication of microdevices for biological analyses have certain requirements including working temperature, sterility, bonding, gas-permeability, transparency, toxicity, etc. For example, even if a device is particle-free (e.g. dust), it might not be sterile in the sense of contamination-risks affecting a PCR or cell culture (e.g. bacteria). Manufacturing often involves extreme conditions, for example strong acids (H₂SO₄, sulfuric acid), high temperatures (500 °C), and voltages (1500 V). Of course, most organic molecules are not stable at such conditions and alternative routes for fabrication might be considered. One alternative is to finalize structures before cells or molecules of interest are introduced. Another is to adapt the manufacturing to biological conditions. The elastomeric polymer polydimethylsiloxane (PDMS) is perhaps the most commonly used biocompatible material for soft-lithography fabrication (Whitesides et al., 2001).

The papers presented in this thesis all have a common denominator in the microwell plate consisting of silicon and glass (Figure 2) wherefore the relevant parts of microfabrication is briefly touched upon in this chapter and described in more detail elsewhere (Li, 2006). Hundreds or thousands of through holes are etched by potassium hydroxide (KOH) in a silicon wafer (thickness, 500 μm), using standard photolithography techniques. The wells are created when the array of through-holes in silicon meets the glass (thickness, 175 μm – 1000 μm) by standard anodic bonding. Some advantages with silicon and glass are the rigid well structure, the surface chemistry, the tight (non-leaking) bonds and the reproducibility in-between devices. Compared to PDMS, silicon is not an elastomeric and flexible material and can thus be stored for a long period of time without altered properties. Transparency, and
flatness are the major cause for the sandwich design of the plate, offering a robust and even surface for cell growth and imaging. For cell-based applications, a PDMS membrane is reversibly placed on top of the plate, sealing the individual wells, minimizing evaporation and providing gas-permeability. PDMS elastomer is mixed (according to standard protocols) with a curing agent in different ratios (e.g. 1:10) depending on the rigidity of the final structure, and cast upon a master (e.g. silicon structure) for curing (e.g. 2h, 70 °C) and autoclaving (e.g. 20 min, 121 °C) before use. The mixtures and equipment needed are inexpensive, and the properties (e.g. transparency, gas-permeability) make PDMS a convenient material for many applications.

Figure 2. Photograph of a microwell plate used in the studies presented in this thesis. Standard microtiter-format (128×86 mm² holding 3243 wells) for instrument compatibility. Scale bar 3 cm.
2\textsuperscript{nd} chapter: Single-cell analysis.

By studying one cell at a time, the results are certain to stem from that particular cell. A cell can for example be monitored as it migrates or divides into two cells, how many divisions that occur over time and the rate of cell division in a specific cell clone. Cell divisions are interesting in for example cancer, due to the uncontrolled rate of cell proliferation of a cancer cell as compared to a normal cell. Genetic patterns and protein expression can explain cellular behavior to a large extent, but the dynamic study of living cells can increase the understanding of the interconnecting molecular events continually taking place in each cell. Each cell is more or less different from the other, even within the same cell type (Spudich and Koshland, 1976). Cellular heterogeneity is well known in bacteria and increasingly apparent in eukaryotic cells (Templer and Ces, 2008). For a long time it was desired as well as assumed that cell cultures were of a homogeneous nature, and that analyzing a collection of cells would give an accurate assessment of the behavior of the cells in that culture or tissue. The average response of the cells was, and often still is, interpreted as the response of all cells in that sample. Additionally, the effect of cell number (signaling, interactions etc.) on cell behavior was, and is still often, neglected due to difficulties in monitoring such detailed phenomena. The attitude to ensemble measurements is starting to change and today's researchers are increasingly aware of, hence interested to study, the resulting effects of heterogeneity in cell samples, as mirrored in recent reviews on single-cell analysis (Andersson Svahn, 2007; Di Carlo and Lee, 2006; Longo and Hasty, 2006; Sims and Allbritton, 2007; Voldman, 2006).

This chapter discusses the benefits of analyzing single mammalian cells as compared to averaged ensemble measurements, and briefly touches on conventional methods available for the study of individual cells.

Single cells as compared to ensemble analysis.

It is well known that individual cells differ from each other in many aspects and the way cells process input signals decides the fate of that particular cell. Heterogeneous behavior of cells, such as molecular actions and signaling, are known to occur under identical environmental conditions (Rao et al., 2002; Raser and O'Shea, 2005). This can be visualized by variations in cell division, drug efficacy, cell cycle stage or age,
phenotype due to the stochastic nature of gene expression, receptor expression, concentration of a critical metabolite or ion as demonstrated in a few examples from the last decade (Blake et al., 2003; Diercks et al., 2009; Elowitz et al., 2002; Ferrell and Machleder, 1998; Marcus et al., 2006; Teruel and Meyer, 2002).

There are many reasons why individual cells respond differently to a given stimuli and the rich information obtained from single-cell analysis can result in better understanding of the molecular machinery of a cell and its role in a complex environment. An analogue with drug development describes the approach; if a group of patients with a given disease X are given the drug A, only ca. 50% of them will respond to the treatment. The rest of the patients will experience nothing but unwelcome side effects. By looking into individual patients and identify the responders (and non-responders), a better treatment can be offered. Sectioning patients on their responding behavior rather than by the name of their disease, is what is popularly called personalized medicine or personalized healthcare (PHC), long envisioned and now approaching reality. Visionary statements of single-cell analyses as potential techniques for progress in PHC can, similarly to the above example, be explained as follows: if a million of cells are cultured in a conventional Petri dish and treated with a drug, the outcome of the cell response is an average response from all the cells within the dish. Thus, it is impossible to find out whether the effect results from a small, homogenous response from all cells or a large response from a subset of cells. The most probable situation is a wide range of responses where some cells do not respond at all, as opposed to other cells. Yet most cell-based biological assays yield averaging data from large groups of cells. As discussed by Di Carlo, Wu and Lee (Di Carlo and Lee, 2006), average responses from bulk samples can hide data and even be misleading. Single-cell analysis on the other hand offers dynamic information of individual cells, schematically shown in Figure 3. Using leukemic cancer cells as an example, if the number of cell divisions of a group of cells in an ensemble is determined to be 20 (Figure 3a, average value in brown), the true case might be more of an on/off behavior, due to proliferation-differences among the studied normal (Figure 3a, yellow) and abnormal cancer cells (Figure 3a, red). The two groups of cells would in a single-cell assay give rise to two separate proliferation-rates, namely 0 and 40. Or even likelier, the outcome 0, 10, 30, and 40 due to four subgroups of cells, as shown previously on mixed cell populations from tumor biopsies where a significant heterogeneity exists even within the group of abnormal cells (Bodey, 2002; Fink et al.,
Furthermore the onset time for cell division differs dependent on cell-cycle variations, e.g. some cells divide almost instantly while others divide later (Figure 3b). Additional possibilities are that the first division (resulting in two cells) is instantaneous while the second cell division (resulting in four cells) for some reason(s) is postponed, or that cells start to die resulting in a decreased clone size (Figure 3c). Using ensemble technologies, the conclusion of such an experiment would be that all cells divide linearly but slow (Figure 3b,c -average value in brown), and details of cell variance would be overlooked. Subsequently, the purpose of single-cell biology is to increase knowledge and understanding at the level of individual cells, in order to better understand the entire human body. Heterogeneity in cell populations has an important biological role, interesting also from an evolutionary point of view.

![Figure 3](image.png)

Figure 3. Illustrations of cell heterogeneity vs. ensemble analysis. (A): Two groups of cells, e.g. normal (yellow) and cancer (red) cells, would in a bulk assay yield misleading average response (brown) on the number of cell divisions on a given cell sample. (B): Differences in onset time of cell division between single cells (yellow and red lines) would be interpreted differently in a bulk assay (brown). (C): Dynamic information of cell division over time (e.g. cell death) can be monitored using single-cell analysis (yellow and red lines) as compared to bulk analysis (brown).

Still, many researchers employ bulk methods for cell analyses because they are uncomplicated and well-established techniques for assessing intracellular molecular events. The data interpretation is often straightforward, with fewer data points and less need for re-thinking and automation, as compared to newer single-cell technologies. In some areas of research, e.g. drug development the added value of
heterogeneity studies is yet to be realized and ensemble analyses are still employed as the golden standard for cell-based efficacy studies. In other areas, the interest in and potential of single-cell techniques is considered high, but people have problems in connecting and translating their own research into the technology (even if they in one way or the other already study heterogeneity). Once again, the connecting link between technology and biology is decisive for a new method/technology to take off.

A common caveat for single-cell approaches is that cells need interaction and signaling with other cells to function normally and without such conditions, cells will subsequently alter their normal functions including diminished viability. In some aspect that holds true, far from all cell-based assays and/or applications are suitable for single-cell analysis. Yet, studies have shown great potential of analyzing single cells with maintained functions such as cell division, which suggests single-cell analyses as a complementary tool to ensemble analyses. In cell-to-cell communication studies the thought of monitoring signaling between a few single cells is appealing but impossible to realize using bulk methods. Single-cell technologies on the other hand, are well suited for such studies due to the better control of individual cells and their behavior that they offer. Thus, the advantages of single-cell analyses are plenty and as user-friendly techniques become more readily available, the uses thereof should increase.

Petri dishes, test tubes and microtiter plates will probably remain the mainstay method, before target communities add new experimental platforms for complementary single-cell studies. Since technology-development often (and wisely) is not considered an advantage per se by biological researchers, the main drive for approaching a new technology is significant improvements in the analyses. Hence, added value such as enabling research previously thought not to be feasible, is the main reason for applying single-cell analyses as seen from a biologist’s point of view.

Conventional methods.

Single mammalian cells are fragile, small, and have strict requirements of their microenvironment to maintain life (e.g. oxygen, temperature, nutrition, etc). By nature, cell lines are more tolerant and thus easier to work with, compared to primary cells. When analyzing single cells it is important to study many single cells simultaneously in order not to draw misleading or wrong conclusions from rare cells or stochastic biological noise. How many single cells must then be experimentally investigated to acquire adequate sets of data in order to safely draw conclusions? This question has
no general answer since it depends on the application. Conventionally, 10 000 cells is considered a standard number (Givan, 2001) and it has been suggested that aiming for 1000 cells might be enough when developing general single-cell technologies (Andersson Svahn, 2007). The importance of analyzing a large number of individual cells and determining the distribution of responses, due to cell heterogeneity, is undisputed and have been highlighted previously (Mettetal et al., 2006; Yu et al., 2006).

The most commonly used method for single-cell analysis is flow cytometry (FC), developed in the late 1960’s. Flow cytometric technology origins from the three strands of microscopy, blood cell counting instruments, and ink jet technology developed for computer printers. FC allows hundreds of thousands of individual cells per minute to be analyzed according to their size, granularity and fluorescence properties in a wide range of applications, e.g. viability, protein expression and localization, gene expression, etc. (Villas, 1998). Cell sorting, popularly called fluorescence-activated cell sorting (FACS —in fact a brand name owned by BD Biosciences), enables sorting one or several populations of cells from a mixed sample, for further analysis (the sorted cells are normally collected in test tubes or in 96/384-well plates). FC is a “state-of-the-art” technique for well-characterized distributions of individual cell behavior at high throughput (up to 10 000 cells/s). The reason for its success is the throughput combined with fluorescent labeling, allowing semi-quantitative determination of for example various protein levels in a population of cells (Krutzik and Nolan, 2006; Nolan and Sklar, 1998). It is also possible to analyze cells in a time-dependent manner, where cells are sampled at different time points (Martin and Swartzendruber, 1980) by FC. However, time-dependent studies on individual cells using FC are not commonly performed since the analyzed cells often go to waste or are sorted (i.e. often mixed) with other cells. Conventional FC require many cells for analysis (at least 100 000 cells) and cells must be mixed again (thereby lost track of) before a second round of analysis, hence FC gives information of the distribution of a group of cells. Using FC it is not possible to follow single cells over time. Likewise, tracking of cell divisions using FC is performed in bulk (Roostalu et al., 2008) and FC was neither designed for handling, manipulation, and dynamic analysis of single cells nor observation of spatial localization of fluorescence within a cell.
Microscopy, another frequently used method for single-cell analysis, is well suited for intracellular localization- and time-dependent studies, and the field of live-cell imaging is built upon monitoring individual cell behavior. The use of microscopy in biological research was introduced in the mid 17th century and is today used on a daily basis in most biological laboratories. As discussed previously, the importance of studying large numbers of cells with accurate interpretation, have resulted in the field of automated microscopy (AM), also referred to as high throughput microscopy (HTM), image cytometry (IC), screening, cellomics, imaging, high content screening/analysis (HCS/HCA) (Oheim, 2007; Pepperkok and Ellenberg, 2006). Normally, cells are either fixed or studied dynamically, and hundreds of images are obtained in a rapid manner followed by massive image analysis to extract useful information about the cells and their intra-cellular compartments. Substantial limitations of AM involve throughput, multiparametric assays, and time spent on data acquisition. AM is often used to study average cell behavior but also holds the potential for obtaining results on the heterogeneity of cell samples, though often lacking controlled seeding patterns and identification of cell boundaries for rapid analysis. It is often cumbersome to monitor and track cell divisions, since suitable methods for isolating single cells or clones have not been routinely used. The conventional tools (e.g. 96/384-well plates) used for AM are relatively big as compared to the size of a cell. More importantly, the wells of a multiwell plate are unnecessarily large as compared to the area covered by the objective’s “field of view” (even when using low magnification optics), sometimes making AM in combinations with conventional tools less suited for monitoring individual cells.

Examples of other techniques for single-cell analysis are i) Laser scanning cytometry (LSC) which allows imaging and quantitative analysis of individual cells in tissues in situ (Harnett, 2007), ii) Capillary electrophoresis (CE) for efficient separation and sensitive detection of whole cell or subcellular samples (Arcibal et al., 2007), and iii) Laser capture microdissection (LCM) for cutting out and separating single cells from tissue for further analysis, such as gene expression and protein analysis (Kehr, 2003). The major drawback for all three techniques is the throughput, and for some kinds of analyses the above-described techniques are not suited. There are several applications lacking good tools for single-cell analysis, for example investigating perforated or encapsulated cells, cellular behavior and cell-cell interactions in microengineered environments, as nicely reviewed recently (Sims and Allbritton,
Additional novel applications are most certain to appear as appropriate tools for robust single-cell analyses become increasingly available.
3rd chapter: Single-cell analysis on chip.

Microfabrication is in many aspects well suited for shaping tools intended for cellular analyses, not least due to the size-compatibility. By elegant means, microenvironments are created to resemble the complexity of in vivo conditions (e.g. 3D surroundings) enabling detailed studies of the complex machinery of cells and their response to certain stimuli. Microdevices for examining drug effects, small molecule and toxicology screens on living cells together with the well-explored ability to integrate several steps of analysis onto a single chip enable novel experimental designs. The countless number of cell-based applications include culturing, electroporation, separation, protein localization, live-cell imaging, drug screening, adhesion, sorting, patterning, trapping, patch clamping, transfection, lysis, microenvironmental control etc. Miniaturized devices for cellular experiments and cell-based assays have been developed for a long time and some well-cited early reviews (Kane et al., 1999; Quake and Scherer, 2000; Whitesides et al., 2001) as well as more recent reviews (Andersson and van den Berg, 2004a; Dufva, 2009; Fernandes et al., 2009; Paguirigan and Beebe, 2008; Tanaka et al., 2007) clearly demonstrates that cell-based research on chip has expanded dramatically in the last decade. More recently, and the main focus of this thesis, the study of individual cells has gained increased attention in the field of miniaturization.

A single cell weighs a few ng, has a volume of ~1pl, a size of ~10 μm and consists mainly of water (Cooper and Hausman, 2007). Inorganic ions and small organic molecules (sugars, vitamins and fatty acids) make up most of the cellular content, and less than 25% (by weight) are made up by proteins and DNA/RNA. The molar concentration of a gene is only in the order of 10^{-12} M while the total protein content is as high as 10^9 molecules per cell (hundreds of pg). It has been estimated that a cell contains more than 100 000 different proteins, ranging from <100 copies of many receptors, 1000 – 10 000 copies of signaling enzymes, to 10^8 copies of some structural proteins, according to the book “The Cell”. With those basic facts in mind, the suitability of LOC devices for single-cell analyses can be better understood. Common guidelines for analytical single-cell techniques are the aim of time-efficient, systematically arranged and accurate analysis (often referred to as high-throughput), instrument compatibility, transparency (imaging/optical properties), cell accessibility
(manipulation and handling), stability and robustness (cell tracking), sensitivity, mimicking of *in vivo* conditions, user-friendliness, and gentle cell handling. Since there are so many and widely applied methods and devices for single-cell analysis on chip, this chapter aims to serve as an overview of available techniques by showing some illustrative examples and discussing applications and important aspects, rather than aiming for full coverage of the field. The section named “microwells” is the most significant section with regards to the papers upon which this thesis is based and thus discussed in more detail than techniques mentioned in other sections. Finally, a short summary from an applications point of view is provided.

**Background.**

Methods for single-cell analysis have been developed in laboratories worldwide for a long time, yet additional solutions are needed. Attempts include miniaturization and mimicking of conventional instruments for single-cell analysis, as well as exploration of novel ideas and techniques that enable new types of experiments. As the scientific field of “cells on chip” continues to grow, “single cells on chip” has started to increase during the last years, as visualized in Figure 4.

![Figure 4](image-url)

*Figure 4. Diagram showing the increased number of publications on chip-based cell analysis together with the emerging publications on single-cell analyses on chip.*
The increased interest in single-cell analysis in general is also mirrored by the funding raised for centers and collaborative projects (Single Cell Analysis Project), meanwhile books (Anselmetti, 2009), new journals (OAP: single cell analysis) and special issues, conferences, and workshops devoted for the topic are emerging. Efforts in research logically also evolve into “single-cell”-related patent filing (Patents Online) and commercialization of technologies resulting in start-up companies and more established companies offering devices for user-friendly analysis of individual cells (AmpliGrid slide; CellScribe™ Population Array; CKChip; LiveCell Array; Picovitro plate) to mention but a few. The analysis of individual cells on chips has also extended into subcellular analysis (Arcibal et al., 2007; Olson et al., 2005) and even detection of single molecules (Jarvius et al., 2006). Moreover, microfluidic patch-clamp techniques for the control and analysis of the intracellular environment of single cells (Olofsson et al., 2009) is a welcome complement to the many methods for controlling the extracellular environment. Instead of looking deeper into the single cells, the field of tissue engineering aims for the whole picture of understanding of how single cells are connected in functional tissue, another promising area for chip-based research (Andersson and van den Berg, 2004b; Khademhosseini et al., 2006; Mikos et al., 2006; Tsang and Bhatia, 2007). Additionally, impressive studies have shown the use of microsystems for studying single bacteria cells (Cai et al., 2006; Elf et al., 2007; Huang et al., 2007). The focus of this thesis however is limited to mammalian single-cell analyses.
Microwells.

A common method to isolate individual cells is to mechanically separate the cells by physical boundaries, i.e. wall structures. Conventional plastic multiwell plates are built on the idea of parallel analysis of multiple samples, why microwell fabrication in that sense can be regarded as “mimicking and improving by miniaturization” from standard multiwell plates. It is a simple and straightforward technique, in terms of theory, practical handling and fabrication. Depending on the intended application, a microwell device can be designed in numerous different ways by selecting different parameters such as shape, size, number of wells etc. as exemplified in Table 1.

Table 1. Overview of a number of parameters in microwell chip designs.

<table>
<thead>
<tr>
<th>References</th>
<th>Shape of wells</th>
<th>Material of wells</th>
<th>Size of wells</th>
<th>Number of wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tight, 2000</td>
<td>Square</td>
<td>Glass, Silicone</td>
<td>&lt;30 µm, ca. 50 µm, &gt;100 µm</td>
<td>90% 10 000s 100 000s</td>
</tr>
<tr>
<td>Chin, 2000</td>
<td>Hexagonal</td>
<td>PDMS, Fiber, PEG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barany, 2005</td>
<td>Round</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betti, 2005</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dastan, 2006</td>
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<tr>
<td>Tokmakov, 2007</td>
<td></td>
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</tbody>
</table>

Well sizes and densities.

By spatially arranging single cells, cell population heterogeneity can be experimentally explored, rather than interpreting cell to cell variation as noise. Larger wells, harbouring many cells, commonly offer long-term culture (days, weeks) while smaller cell-scalable wells are designed for instant analysis (hours, days). An overview of a few microwell designs is given in Figure 5. An early example of cell-sized wells was the high-density optical microwell array for live-cell biosensing, presented a decade ago (Taylor and Walt, 2000). Simultaneous and repetitive measurement of viability (~20 h) and pH dependency of hundreds of individual mouse fibroblast cells, were obtained by fluorescence signals guided to a CCD camera via optical fibers. The array was fabricated by performing a wet etching process on a bundle of hexagonally packed fibers, resulting in fluorescence detection as the only possible read-out method. The well diameter of 7 µm, i.e. smaller than a prolonged cell, resulted in observations of cells moving in and out of the wells.

The number of wells on a chip is often a function of the size of each well, and high numbers of wells are often required for accurate statistical data interpretation. Being able to study the heterogeneity of single-cell responses when treating the cells
similarly have resulted in many useful devices with cell-sized microwells (Figure 5a) (Deutsch et al., 2006b; Kurth, 2009; Tokimitsu et al., 2007). Well-shaped structures of 10 and 20 μm in diameter have been fabricated using PDMS stamping of PEG poly(ethylene glycol) onto silicon substrates for culture of single mouse fibroblasts (Suh et al., 2004), and polystyrene substrate for single epithelial cells (Dusseiller et al., 2005). Mid-sized wells (Figure 5c) have been fabricated by surface engineered PEG on glass, creating arrays for improved optical cell imaging with wells capable of harboring more than one cell, such as 30x30 μm (Revzin, 2003) or 15x15 μm (Revzin et al., 2005) wells. Larger wells (100 μm) was utilized in a cell-retaining PDMS structure presented by Deutsch and colleagues (Deutsch et al., 2006a). Another example using larger wells (tunable from 20–500 μm, Figure 5d) is a platform developed for studying stem cell fates by Chin and co-workers (Chin et al., 2004). The larger wells facilitated long-term (days to weeks) analysis and AM was used for efficient imaging of the ten thousand wells.

Figure 5. A chosen set of microwell approaches, described in detail in (A): (Tokimitsu et al., 2007) close up of one well with a diameter of 10 μm, (B): (Rettig and Folch, 2005) d=25 μm, (C): (Deutsch et al., 2006b) d=20 μm, (D): (Chin et al., 2004) side=100 μm, (E): (Revzin et al., 2005) close up of one well with a side of 15 μm.
Material considerations.

A wide range of materials has been used for well-based single-cell chips. Silicon (Tokimitsu et al., 2007) and glass etching/drilling (Deutsch et al., 2006b) are standard approaches where slow chemical grinding and polishing can adjust the required smoothness of the surface for cell contact. Cell behavior as a result of surface roughness is one example of suitable studies that can be performed using microfabricated devices. Also, the effects on single cells depending on the size and shape of a microwell are interesting features not commonly studied. For applications including imaging, a transparent material with good optical properties is preferably used, such as flat and thin glass surfaces resembling conventional cover slips for microscopy. The additional fabrication step of bonding two surfaces together for smooth surfaces, instead of direct drilling or etching, might be beneficial for some applications and is frequently used. Another material commonly used for the formation of well structures (Figure 5b) is the “biologically inert” polymer PDMS (Ostuni et al., 2001; Rettig and Folch, 2005). PDMS-wells have for example been applied to 3D cell shape control of single cells (Ochsner et al., 2007). Since PDMS is an elastic material, effects of molecular diffusion into and out of the material as well as other biological implications need thorough investigation (Regehr et al., 2009). Its flexibility is often considered an advantage, e.g. facilitated sealing onto other materials, but can sometimes be a disadvantage, e.g. collapse of thin walls (Delamarche et al., 1997). Beside the mechanical properties, the optical properties need to be taken into account. Material auto-fluorescence and disturbing (as regards to the function of the device as well as to the field of view) bubble-formations due to the hydrophobic nature of PDMS can occur. As for any material the advantages and disadvantages depend on use.

Wall design.

In general, a chip design with low walls and small wells is suitable for spatially ordering single cells when signaling and cross-contamination between the cells and wells are allowed or intended. Evaporation problems often arise in small wells due to the small volumes (e.g. 85 fl. in Taylor et al.) and a common solution to that problem is the use of a large volume of culture medium on top of the array, i.e. shared analytic solution between all wells. This approach can be beneficial in some applications while problematic in others. Chip designs in which the walls are lower than the cells’ height
Microwell devices for single-cell analyses

(Figure 5c), utilize the advantages associated with shared solution between the wells (Deutsch et al., 2006b). Moreover, wall heights are not only important in setting well volumes but also holds a protecting and shielding function for cells under harsh conditions. Cells are held in place with very low forces (if any), which causes less stresses on the cells, as compared to cells in fluidic devices or on open surfaces. Toner and colleagues describe this phenomenon by freezing experiments where unprotected cells, i.e. cells attached to an array but not in a well structure, were lysed or detached from a surface during preservation (Roach et al., 2009). Similar tendencies can be imagined in cell staining experimentation and other protocols involving tough rinsing or mechanical affect on cells, although this remains to be demonstrated.

**Cell types and cell seeding.**

It has been more challenging to generate microdevices for the analysis of single non-adherent cells (such as blood cells) than for adherent cells, due to the difficulty to retain non-adherent cells at a known location during manipulation of the device. Most previous work has thus been performed on adherent cells, but as more tools for single-cell analyses are developed, the availability of non-adherent cell analysis on microwell chips have increased.

Suspensions of single cells are normally seeded manually into the microwells, and the cells are randomly positioned in the wells on the chip by gravitation. Remaining cells outside a well are then flushed away, sometimes followed by another round of cell loading to increase the final number of captured cells. Parameters to keep in mind when evaluating single-cell chips are seeding efficiencies such as i) well occupancy, i.e. how many wells contain cells and ii) single cell occupancy i.e. how many wells contain single cells. Often, since so many wells (usually hundreds or thousands) are offered it is not critical that all wells are available for analysis. Trapped air bubbles, evaporation and pieces of dust are common obstacles that diminish the number of wells accessible for analysis. Chemical treatments can enhance seeding efficiency by providing repelling surfaces on the walls contra attractive surfaces inside or at the bottom of the wells (Revzin et al., 2005). It is difficult to set a general limit of the acceptable ratio of cell seeding but if less than ca.70% of all wells in a device can be analyzed after cell loading, the device might not be optimal for that type of analysis considering the time taken for analysis. Still all wells require detection including the failed ones (that after a first round of detection are ignored for continuous detection).
Cell retrieval.

Cell manipulation and handling are often facilitated when working with larger wells, since spatial access with micromanipulators or bulkier tools is offered. PEG-on-glass structures (Revzin, 2003; Revzin et al., 2005) have been used as cytometry platforms for analysis of leukocytes, mouse fibroblasts and primary rat hepatocytes, and LCM mediated retrieval of selected single cells from the platform was shown, useful for further genomic or proteomic analysis. Another example of cell retrieval from cell-sized wells demonstrates the picking of positively detected antigen-specific B-cells using micromanipulation (Tokimitsu et al., 2007). Cell retrieval and/or removal are critical parameters for microwell chips in general, i.e. can the cells be retrieved post analysis or are they lost? Many single-cell chips are designed for continuous analysis on chip without the possibility for the investigator to retrieve interesting cells or clones. However, techniques for retrieval and manipulation of cells are very important, since screening attempts often end up in particularly interesting findings regarding only a few cells which it would be desirable to in one way or another analyze further. Most of the existing techniques for cell picking and retrieval are compatible with open well structures and thus can be combined with most microwell-based assays whenever necessary.

Detection systems and dynamic read-out.

Many high-density well chips have been integrated with suitable detection systems for rapid and accurate data interpretation. Well structures, i.e. where cells are held still in one position as compared to fluidic devices, are often suitable for bright field and fluorescence analysis, leading to high-throughput screening as a common goal. For instance, one way of providing automated and rapid detection to a cell chip, was the modification of a DNA chip scanner by replacing a laser to improve maximum resolution (to 2.5 μm), resulting in a cell scanner suitable for single-cell analysis (Tokimitsu et al., 2007), later on modified for improved time-lapse monitoring in the same device (Ozawa et al., 2009).

A general drawback with microwells is the sometimes-lacking functionality to measure dynamic rapid cellular responses as a result of instant interaction with a reagent. Since reagents often are distributed across the entire well chip, difficulties in practical liquid dispensing might collide with the requirements for simultaneous detection. In many cases, the different wells cannot be individually addressed with
different solutions due to risk of cross-contamination. In cell heterogeneity studies using cell-sized microwells, cross-contamination is seldom a problem, since such chips are intended for exploring the variance in single-cell response under set conditions. However, microwell chips are also useful in applications such as drug efficacy screening where different wells need to be more or less separated and addressable. Although the cells might be spatially separated, good methods to address the exact same cell at several time-points are required in order not to lose track of a particular cell during the different measurements. Keeping track of the individual cells’ positions can either be done directly on the chip/microwell, or by obtaining exact coordinates of each well in the detection system.
Patterns.

Creating other arrays than wells, by micropatterning of surfaces, is a frequently used technique for spatially arranging single cells for analysis, as demonstrated by the examples shown in Figure 6. Patterning is a wide-ranging term, often referring to spots on a surface for directed cell adhesion and growth but can also comprise patterning of polymers, such as PEG, creating well-shaped structures. In this chapter, the latter are discussed in the previous section; “microwells”.

The general concept of micropatterning is to provide cytophilic (cell-friendly) vs. cytophobic (cell-repelling) regions by modified surface chemistry of a substrate. Two examples of commonly used cytophilic extracellular matrices are fibronectin and laminin, whereas PEG is often used for its cytophobic properties. Photolithography has been an extensively used technique for patterning of cells, and a decade ago the complimentary technique soft lithography became frequently used for microcontact printing and micropatterning, reviewed by Whitesides and colleagues (Kane et al., 1999). Ideally, micropatterning should offer dynamic surface properties and cell patterns, with abilities of connecting different cell types for interaction studies and building 3D networks, as reviewed more recently by Voldman (Voldman, 2006).
Adherent cells, as compared to non-adherent cells, have long been considered more available to study in array-like formats due to the possibility of positioning the cells, allow them attach to a particular spot/area and then keep track that area during cell growth for analysis (Figure 6a). Surface coatings and patterning can be modified to optimize conditions for each microdevice. With new robotic spotting technologies volumes of nanoliters can be dispensed to surfaces or cells in an addressable way, opening up for a wide range of patterning approaches. Attempts of steering and capturing cells to surfaces in non-natural manners have been conducted using e.g. engineered cell surface receptors (Kato and Mrksich, 2004), magnetic nanowires (Tanase et al., 2005) and DNA hybridization (Chandra et al., 2006). In most in vitro assays the potential biological effects on the cells must be considered when engineering substrate surfaces, in order to maintain cell viability for proper experimental conclusions. The main advantages of tethering cells are that fluid can be passed over the cells, supplying the cells with nutrition and/or reagents, and that cells can be positioned in any arrangement and manipulated in a chosen manner. Potential drawbacks needing consideration are flow-induced forces and their biological effects, e.g. shear stresses.

Dynamic substrates that change their cell adhesion in response to external stimuli such as heat, voltage and light has been recently reviewed (Nakanishi et al., 2008). Kaji et al. presented a dynamic cell patterning method using an electrochemical probe to turn cytophobic areas cytophilic by desorbing the blocking agent bovine serum albumin from a substrate. The cells are first kept to a stripe and next allowed to migrate off that stripe (Figure 6c) and onto new areas (Kaji et al., 2004). Thus, by locally changing substrate adhesion a dynamic cell pattern can be created, for patterning of multiple cell types and creation of dynamic surfaces. Cell signaling is another suitable application for micropatterning techniques, for example a neuronal network in rat hippocampal cells has been formed in channels created by photothermal etching (Sugio et al., 2004). In a time-dependent manner, cells can be allowed to interact via physical contact (Figure 6d), provided by altering structures during the experiment.

Micropatterned surfaces for controlling cell size, growth, and morphology have been widely studied. The direction of cell migration (Jiang et al., 2005) and cell division (Thery et al., 2005) using asymmetric micropatterns showed the possibility to investigate cell-material interfaces and the cellular behavior resulting from interactions
at these. Furthermore, cells have been switched from growth to apoptosis by
decreasing the area for cell growth (Figure 6a), progressively restricting cell extension
and obtaining geometric control of cell growth and viability (Chen et al., 1997).

Arraying single cells in high-density patterns for screening applications is a
commonly used technique similar to microwell approaches. An early example was the
use of elastomeric membranes with circular or square holes for lift-off patterning on
e.g. conventional Petri dishes (Ostuni et al., 2000). More recently, clonal assays for
high-throughput screening of stem cells were performed by Ashton et al. Cytophillic
spots were made by microcontact printing. Subsequently seeding of single rat
hippocampal neural progenitor cells directed the forming of neurospheres (NS) on a
microarray (Figure 6b). The NS were then retrieved (here, pipetting of NS is the
bottle-neck from a time-perspective) for conventional expansion in larger culture
volumes. Since NS normally grow in suspension and are difficult to keep track of (and
maintained apart without merging), the demonstrated method of arraying NS has high
potential in the field of stem cell research (Ashton et al., 2007).
Traps.

Similarly to well- and pattern approaches, trapping of single cells at fixed positions facilitates maintenance of the cells for analysis during longer periods of time (as compared to cells passing in a flow). Trapping is often combined with microfluidic systems, providing measurements on short time-scales and continuous supply of nutrients or reagents, occasionally offering “trap-and-release” functions. In microwell- and patterning approaches the cells are (at least somewhat) in contact with a surface. There are both contact and non-contact mode trapping techniques, reviewed recently by Laurell and colleagues (Nilsson et al., 2009). Examples of a few trapping techniques are shown in Figure 7.

![Figure 7. A chosen set of trapping approaches, described in detail in (A): (Di Carlo et al., 2006), (B): (Lin et al., 2009), (C): (Zheng et al., 2007) diameter of hole: 10 μm, (D): (Shi et al., 2009). Scale bars 50 μm.](image)

**Mechanical traps.**

Trapping is sometimes accused of being low in throughput, but there are examples demonstrating the opposite. Lee and colleagues created uniform arrays of trapped single cells in a microfluidic device (Di Carlo et al., 2006). U-shaped mechanical/hydrodynamical traps (Figure 7a) are exposed to a change of the surrounding flow after cell loading, resulting in a time-efficient cell loading of empty traps (ca. 30 s). The device can be used for studying diffusible secretions of single
cells, and is compatible with standard microscopy. It is not designed for long-term analysis (maximum ca. 24 h) and the risk of cell loss, due to the flow, need to be considered. The cell retainer represents a convenient way of arraying single cells for the dynamic short-term study of rapid responses to various stimuli. The U-shaped trapping structures were developed further by the Voldman group to offer controlled pairing of two cells in an elegant and high throughput manner (Skelley et al., 2009). Centrifugal forces in CD-like laboratories is another example of mechanical trapping of single cells (Lee et al., 2008).

Magnetic traps.
Many single-cell chips are limited to analyzing all cells within a population, with no possibility of choosing a subset of particularly interesting cells to study. An array of magnetic traps (Figure 7b), generated by magnetic flux density peaks at predefined locations, shows the capture of a selection of cells by immunomagnetic labeling (Liu et al., 2009). To facilitate cell interaction with the magnetic fields, cells are labeled with antibodies coupled to dextran-coated nano/micro-magnetic particles making it possible to sort out certain cells based on e.g. their cell surface protein expression. By linking markers for cell heterogeneity (such as surface expression) to magnetic properties, useful single-cell tools for short-term analysis are created. Magnetic particles have also been inserted in living cells, in order do control and direct a trapped cell (de Vries et al., 2005) and magnetic methods have been used for trapping non-magnetic objects such as several different cell types, by the use of a particular surrounding buffer (Winkleman et al., 2004). However, the biological effects of coupling cells to beads or particles need consideration, in this type of experiment of application.

Hydrodynamic traps.
The most common method for trapping cells in microfluidic systems is to create side channels to a main transport channel, where the side channels are small enough to trap cells by suction when a minor part of the main flow is withdrawn that way. Using a similar strategy, hydrodynamic single-cell trapping was shown by the van den Berg group. By the use of microholes for connecting two channels in a silicon-glass microfluidic device, trapping sites for living cells were created. The device have previously been applied for the study of apoptosis dynamics in human leukemic cells
Microwell devices for single-cell analyses (Valero et al., 2005) and more recently in gene transfer and protein dynamics studies of single stem cells (Valero et al., 2008). Aiming for higher throughput, an array with 16,000 circular through holes, each with a diameter of 10 μm (Figure 7c), has been presented for the capture of non-adherent cells (Zheng et al., 2007). The hole-size was adjusted for trapping circulating tumour cells while erythrocytes where allowed to pass through, thus providing a cell-size dependent trap for a particular set of single cells. Furthermore, in this setup, each single cell is placed in contact with electrodes to enable \textit{in situ} cell lysis and subsequent PCR amplification. However, hydrodynamic cell trapping is suited for short-term analysis. For all techniques, potential biological effects of trapping and deforming cells need to be taken into account when performing this type of experiment.

\textbf{Optical traps.}
Focused laser beams, so-called optical tweezers, is one way of manipulating cells with very high precision and possibility to spatially arrange cells. Microscale objects are forced by optical forces towards the focus point of a laser beam, where the trapped objects can be repositioned in all dimensions by moving the beam and changing focus. Prolonged handling times in small volumes can pose a problem, due to the fact that the absorbed laser energy in the water-based buffers may lead to harmful heating of the cells. Newer optical tweezers offer multi-object handling capabilities compared to the original single beam - single trap systems and can be used to array single cells (Grier, 2003). Multiple optical traps can thus be used in cell heterogeneity studies of single cells, recently reviewed by Ramser and Hanstorp (Ramser and Hanstorp, 2009). An earlier example, with lower throughput but which includes sorting abilities, is the model system for automatic cell identification based on size to sort erythrocytes from other cellular components of human peripheral blood (Grover et al., 2001). Optical forces can also be used in an opposite manner to trapping cells, \textit{i.e.} to remove unwanted cells by lifting discriminated cells from a microwell array, as exemplified in a PDMS channel experiment (Kovac and Voldman, 2007).

\textbf{Dielectrophoretic traps.}
Non-conducting, \textit{i.e.} dielectric, objects such as cells can be moved by forces generated in a non-uniform electric field, as utilized in dielectrophoretic (DEP) cell positioning. A dipole is induced in the cell by an electric field. Depending on properties such as
conductivity of the surrounding medium, a DEP force will draw the cell to the field maximum or minimum. Designing DEP trapping arrays in microfluidic devices using microelectrodes is advantageous due to the increased control of heat removal (i.e. by flow) from the cell-capture sites. To individually control many cell positions, the connection to and control of the electrodes increase in complexity with the number of positions. A way to circumvent this problem and to increase the number of cells available for analysis has been shown with a scalable array for trapping, imaging, sorting and release of single cells (Taff and Voldman, 2005). By using a ring-dot electrode geometry organized on a planar array, the number of “chip-to-world” electrical connections was substantially decreased compared to prior designs. Cells were released by grounding two crossing electrodes while keeping the ring electrode active. DEP has, among other applications, been used for miniaturized cell sorting, by labeling cells with particles that differ in polarization response, hence yielding the ability to sort out rare cells at a throughput of 10 000 cells/s (Hu et al., 2005).

**Acoustic traps.**

Ultrasonic standing waves can be used for non-contact trapping of single or agglomerates of cells, offering dynamic control over cell environment by microfluidics during short-term analysis. A standing ultrasonic wave generates pressure gradients that in a liquid medium give rise to forces on cells (Gor'kov, 1962). Negative effects on the viability of trapped cells have been discussed and depend on thermal control in the devices, but so far no indications of reduced viability due to ultrasound exposure have been presented. Nilsson and colleagues verified short-term (15 min) viability for neural stem cells as well as the concept of a 6-h proliferation assay of yeast cells in an ultrasonic trap for single cells or larger cell clusters (Evander et al., 2007) and the Wiklund group kept 2D-aggregates of adherent COS cells viable for 30-75 min in the chip (Hultstrom et al., 2007). The latter group has recently presented an ultrasonic cage for trapping of individual cells (Manneberg et al., 2008). This approach has also been shown to be useful in cell enrichment of very diluted samples. For cell-patterning approaches, a promising acoustic tweezer-technique in which a few bovine red blood cells was effectively patterned (Figure 7d) using standing surface acoustic waves has been presented (Shi et al., 2009). One disadvantage of acoustic cell trapping is the lack of demonstrated long-term viability of cells, resulting in another method currently only available for short-term analyses.
Droplets.

Similarly to analyzing individual cells in compartmentalized microwells, cells can be enclosed in droplets of low volumes (fl to nl), forming micro-chambers for individual reactions. Since each cell is kept within its own separate droplet, isolated from other droplets, the risk of cross-contamination decreases and the cells can be analyzed in a wide range of applications mainly by fluorescence detection. An overview of functionalities in droplet-based microfluidic devices is shown in Figure 8. The small volume of each droplet offers a diffusion-depending mixing of reagents within the droplet. High frequency (Hz - kHz) droplet generators in microfluidic devices form monodisperse drops (Figure 8a) of water in an inert and immiscible carrier fluid (oil). Controlling the number of loaded cells per drop has been a barrier for droplet-based single-cell analysis, due to the stochastic limitations of single-cell loading resulting in ca. 30% of single-cell occupancy (similar to many microwell approaches). A demonstrated way to overcome this limitation has been to evenly space cells in a microchannel (Figure 8b) to make sure that the cells entered the drop generator with the frequency of drop formation (Edd et al., 2008). As with limited dilution in general arraying techniques, empty droplets are often preferred rather than overloading droplets with several cells. The droplets can thereafter be merged with other droplets (Chabert et al., 2005), split in two (Link et al., 2004) or DEP-sorted (Ahn et al., 2006) among other operations.

Figure 8. A chosen set of droplet approaches, described in detail in (A): (Holtze et al., 2008), (B): (Edd et al., 2008), (C): (He et al., 2005) cell-size: 10 µm, (D): (Brouzes et al., 2009). Scale bars 50 µm.
A strong advantage of analyzing single cells in droplets is the ability of rapid detection of cell-secreted molecules due to the low volume surrounding each encapsulated cell. Griffiths, Weitz and colleagues showed an example of such an application by incubating single hybridoma in 33 pL drops, giving rise to secreted detectable concentrations of antibodies after 6 h (Koster et al., 2008). Moreover, an acceptable percentage of the cells (85%) were shown viable during that period of analysis. Crucial steps in droplet-based cell analyses are the encapsulation of single cells, as well as the maintained viability of cells within the droplets. Risks of coalescence, nutrient depletion or the accumulation of toxic metabolites are obstacles that need to be considered before robust analyses over longer periods of time can be achieved. Cells have been cultured in emulsions off-chip for up to two weeks, implying that analyses at different time points are feasible (Clausell-Tormos et al., 2008) and prolonged single-cell reactions are allowed for repeated analysis. The same paper also describes how viable cells after a couple of days are recovered after controllably breaking the emulsions. Such attempts open up for dynamic analyses by making possible a second round of droplet formation for repeated analysis and the recovery of cells for non-droplet use.

Applications of droplet-based microfluidics are numerous and have high potential in high-throughput screening. An early example of cell encapsulation (Figure 8c) demonstrates laser-induced cell lysis within droplets followed by monitoring the activity of the beta-galactosidase enzyme from a single cell (He et al., 2005). The same enzyme was utilized in a signal amplification assay for detection of the low-abundance cell-surface biomarkers CD19 and CCR5 on single U937 cells, using microfluidic droplets and multiplexing (Joensson et al., 2009). Encapsulation permits amplified detection of extremely low levels of biomarker molecules, demonstrating droplet-based microfluidics as a potentially more sensitive method for biomarker discovery than conventional FC. However, the throughput of cell analysis is in general higher in a conventional FC. For example, a recently published paper on fluorescence-activated droplet sorting (FADS) enabled cell sorting based on enzymatic activity at 300 cells/s (Baret et al., 2009) in contrast to standard FC cell sorting instruments that sort cells at up to 10 000 cells/s in a robust manner. After describing separate modules for different purposes, Samuels and co-workers have recently published an integrated droplet-based workflow (Figure 8d) for conducting a mammalian cell cytotoxicity screen at high throughput (Brouzes et al., 2009). Cells were kept viable for 4 days
(though cell proliferation was only detected during the first 24 h) and a drug library was screened for its cytotoxic effect against cells from a myeloid cell line. Thus, droplet-based tools for single-cell analysis appear increasingly interesting for many different applications, mainly due to the potential of high-throughput screening and the benefits of enclosed individual micro-chambers.
Other fluidic systems.

Many of the previously described examples in this chapter are in some way combined with fluidics. However, some single-cell isolation techniques/devices still remain. This fluidic section closes the overview on the various technologies for single-cell analyses. Microfluidic techniques offer a toolbox for the study of individual cells using channels, structures, pumps and valves in combinations for a range of different applications. Compared with static cell culture, fluidics can be used for improved microenvironment control by handling the transport of molecules to and away from the cells under study. Miniaturized fluidic systems also have a high potential in cellular imaging, particularly when utilized as a means of distribution of stimulating factors or reagents. For example, precise control of low-volume delivery of reagents to a single cell (Figure 9a) has been shown in a microfluidic network, capable of passively selecting a single cell from bulk culture (Wheeler et al., 2003). Optical changes in the trapped single cell during sequential reagent-supply were demonstrated followed by monitoring of intracellular calcium ion concentration. Methods for stimulating parts of a cell has also been demonstrated in a microfluidic PDMS chip mounted onto a coverslip and imaged at high resolution (Takayama et al., 2001). The paper demonstrates how liquid behavior in microchannels can be utilized as diffusion is the only mixing process occurring (low) and parallel streams thus flow next to one another without mixing (i.e. laminar flow). Moreover microfluidics enable concentration gradients of a reagent and successively varied flow-rate over cells to study their response (Kim et al., 2006). The ability to parallelize the treatment of cell samples in array-like structures by arranging multiple reagent supplies using certain row and column combinations has shown useful, for example in creating multiphenotype arrays (Khademhosseini et al., 2005) and for analyzing gene-expression in different reporter cell lines (King et al., 2007).
The requirement of analyzing large numbers of single cells in order to statistically assure an accurate representation of the distribution of cell responses and/or behaviors, are enabled with microchannels where small chips can harbor numerous analyses or culturing sites. Cheong et al. measured signaling responses to one or more soluble stimuli and/or chemical inhibitors from thousands of single cells using an immunofluorescence read-out from a high-content cell-screening chip (Cheong et al., 2009). They also evaluate the significance of heterogeneous responses observed in different cell types by showing that NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activity dynamics in individual cells are not asynchronous and instead resemble the dynamics of the population average, in contrast to studies of cells overexpressing p65-EGFP (tumor protein 65 coupled enhanced green fluorescent protein).

Naturally, different microsystem technologies are useful to combine due to their size scales, working conditions, compatible manipulation techniques etc. This chapter has previously described microwell-arrays (Deutsch et al., 2006b) and trapping structures (Di Carlo et al., 2006) as parts of fluidic devices. Additionally, conventional instruments like FC have been replicated in miniaturized systems to provide the same type of analysis while using a less expensive platform. Quake and colleagues demonstrated an early example of a microfabricated cell-sorter where fluorescence interrogation and sorting of individual cells was achieved on an inexpensive platform (Fu et al., 2002) although with lower throughput. This group and others have integrated other techniques, optimized and continued developing microflow cytometry as has been recently reviewed (Ateya et al., 2008). As for all active fluidic
devices, shear stresses on cells as well as other effects on the cells introduced by the method must be taken into account also for microflow sorters.

Many other methods and applications for arraying or investigating single cells using microfluidic technology have been presented to date, e.g. mass determination of single cells on cantilevers (Park et al., 2008), self-loading of cells into mobile 3D microcontainers (Figure 9b) (Leong et al., 2008), open or closed microchannels for neuron growth at low densities (Millet et al., 2007), and cilium mediated cellular responses to flow dynamics (Rydholm et al., 2008). Many technologies are available for single-cell analysis at the gene, protein and cellular level and applications thereof are continually emerging improving our knowledge of and means of studying behavior on the cellular level.
Application summary.

A wide spectrum of methods, devices and technologies for single-cell analysis have been and are being developed and applied, directed towards solving clinical problems. The five technology sections above touched upon the range of applications available for single-cell analyses. This section summarizes the field from a solely application-driven perspective. Single-cell analysis has been applied in intracellular research, gene- and protein content and expression, PCR, cell culture and division, clone formation, differentiation, morphology, lysis, separation, sorting, cytotoxicity and fluorescence screens, antibody secretion etc. In the following part some examples of applied single-cell analysis are provided.

Intracellular analysis of cells, such as amino acid and neurotransmitter content of individual neurons studied by methods such as microcolumn separation (e.g. capillary zone electrophoresis, open tubular liquid chromatography) were early applications with single-cell analyses in mind. The high resolution and small sample requirements enabled quantitative, multicomponent chemical analysis of individual cells (Ewing, 1993; Kennedy et al., 1989). Clark and Ewing also presented the electrochemical study of catecholamine release from single bovine adrenal cells following pharmacological manipulations, using picoliter microvials, at the μTAS conference 1996. Analysis of protein and mRNA content from single cells at a given time point results in large quantities of data on the biomolecular level. One cell is commonly lysed whereby proteins or nucleic acids are separated from the lysate and results can be interpreted. In studies using this approach, differences on single cells compared to bulk samples have been demonstrated (Zhang et al., 2000). Determination of amino acid concentrations (Wu et al., 2004) or fluorescence monitoring of protein expression in a β-gal enzymatic assay (Cai et al., 2006) are other examples of intracellular single-cell studies. Using array-like approaches, cytotoxic effects on HeLa and HL-60 cells have been investigated over a period of a couple of days. The lifetime of the cells and their condition when entering the mitotic phase was correlated to drug exposure, demonstrating the working concept of a cytotoxic single-cell assay (Wu et al., 2007). Other groups have presented results on efficient intracellular delivery of reagents by electroporation on chip (Ionescu-Zanetti et al., 2008). Collection of cell-secreted molecules is commonly being performed, here the advantages of using small formats are obvious as dilution-effects are very low.
Miniaturized systems for studying antibody secretion from individual cells as means of methods for the rapid selection and production of antigen-specific antibodies have shown to be promising alternatives to conventional techniques (Jin et al., 2009; Love et al., 2006). In gene expression studies, single-cell PCR has enabled researchers to distinguish whether two or more genes are co-expressed in the same cell or if the genes are expressed in different sub-populations of the cells (Fink et al., 2006; Ginsberg, 2005). Work towards whole transcriptome analyses (Bontoux et al., 2008; Kralj et al., 2009) are in progress, encouraging the dreams of genetically mapping entire contents of single cells.

Moving from studying the interior of the cell to external stimuli or dynamic cell behavior, microscopic imaging has revealed unique biological phenomena in single cells previously not discoverable using ensemble analysis. The control of cell spreading and the cellular effects of altered cell shape were investigated early on by patterning techniques (Chen et al., 1997), and is today a method contributing to our understanding of cell division and cytoskeletal organization (Thery et al., 2005). Separation of blood cells (Grover et al., 2001) and controlled pairing of cells (Skelley et al., 2009) are other examples of current biological work on individual cells. Many useful applications have been demonstrated for single-cell analysis in microwells. Recently Lutolf and co-workers presented a glass/hydrogel platform for controlling the size of neurosphere formation, by a reliable and non-merging clonal assay. The viability of neural stem cells was shown to increase two-fold, compared to conventional methods using plastic dishes (Cordey et al., 2008). The high-throughput platform should be helpful in elucidating the function of sphere-forming stem/progenitor cells independent of their proliferation dynamics.

Clonal growth of single cells has for a long time been studied in stem cell and cancer research in multiwell formats (e.g. 96-well) and is now also emerging as a field of study in microchip formats. Cell culture and division are commonly investigated. Robust and efficient chip-based methods for long-term studies (weeklong) of single cells are emerging, still, shorter analyses are much more frequently reported. A common methodology is to utilize microscopic readout from arrayed single cells where instant responses are analyzed (Di Carlo and Lee, 2006) after treating all cells with the same reagent. Tools for high-throughput imaging and image processing have been developed for a long time and the use of fluorescence readout is successfully applied in various investigations, e.g. studying stem cell fates (Chin et al., 2004). To
screen a high number of single cells is critical in heterogeneity studies, wherefore low-throughput methods (only allowing the study of 1-10 cells in a reasonable amount of time) are more limited in terms of practical use. The majority of the current work on chip relies on mammalian cell lines. Primary cells have more stringent requirements on their environment for survival, why systems are normally verified with cell lines before moving to primary cells. For clinical applications though, being a common goal, conditions and solutions allowing the robust use of primary cells are necessary but still not commonly available.

Due to the fact that proteins and other analytes of a cell cannot be amplified, in contrast to genetic material, sensitive methods for detection are needed. Methods that are sensitive enough for low level detection of protein- or gene expression, can facilitate the readout of the genetic content of a single cell and also link the information to the cell’s dynamic behavior. To summarize, many useful constituents of assays exist and some “ready-to-use” devices already offer analysis of single cells in many different applications. Still, the field is immature regarding clinically requested assays why efforts in the development of such are needed. However, the broad span of platforms and the flexibility and increased knowledge this brings, implies that we are heading in the right direction and visions such as “portable devices for personalized healthcare” are continually moving closer to reality and practical clinical use.
INVESTIGATIONS
Microwell devices for single-cell analyses
4th chapter: Objective.

This thesis is based on the development of a microwell plate suitable for *in vitro* assays such as cell culture and analysis assays. The work includes technical development as well as biological application development in the field of single-cell analysis.

The initial objective was to develop a single-cell device allowing controlled single-cell seeding. By knowing the exact position of each individual cell, the study of several different cell types on a single device would be made possible. Two main goals were that the device should have “state-of-the-art” optical properties and that it should enable the study of large numbers of single cells in order to carry out diverse heterogeneity studies on cell populations. Clonogenic studies of cancer cells were deemed suitable for proving the biological use of such a device (Paper I). The individual wells of the device would preferably be isolated from each other, in order to enable the study of effects of various reagents on individual cells (from one or several cell samples) at high throughput. Light microscopy was considered the most important method to monitor cell morphology, cell proliferation etc. in the device. Fluorescence microscopy and screening assays (Paper II) were other methods of detection envisioned for the platform. The aim was to keep the well volumes small enough to facilitate experiments with expensive reagents (Paper III) but large enough to enable practical handling of the individual reaction chambers (Paper I-V). Aiming for a device useful in a wide range of biological assays, such as cell culture analysis (Paper I-V), protein analysis (Paper III) and genetic analysis (Paper IV), demanded an open configuration of the device and one that was compatible with standard laboratory equipment. In the choice of materials for the device, future needs and modifications as well as integrations such as heat transfer (Paper IV) or fluidic access to parts of the device (Paper V) were carefully considered.

The scientific work whereupon this thesis is based, started in the year of 2006 and has since moved from the prototype/idea stage into a stage where a useful device for single-cell applications within the fields of cancer, stem cell, protein and DNA research is available. Additionally, technological improvements such as an optimized thickness of the bottom glass layer and integration of fluidic handling have broadened and improved the device.
5th chapter: Results and discussion.

I. A microwell plate allowing single-cell sorting and culture.

In this study a microwell plate was developed to allow single-cell sorting using a FACS instrument, followed by weeklong cell culturing of clonally expanded leukemic cancer cells.

In vitro assays are widely used for assessing cellular responses and understanding the specific response of cancer patients to various drug treatments. Leukemia, a cancer of the blood or bone marrow, is characterized by abnormal cell proliferation of blood cells and commonly studied in clonogenic assays to predict and measure toxicity and efficacy of anticancer drugs and radiation (Blumenthal, 2005). By splitting up heterogenic patient tumor cell clusters into single cells, culturing them individually and studying how clones are formed, cancer cells can be distinguished from healthy cells as they are analyzed. This analysis can be performed in parallel. Furthermore, subgroups within the set of cancer cells can be discovered, e.g. cells with an increased rate of proliferation or a particular response to a drug etc. This is of critical importance in predicting long-term clinical outcome.

The mammalian cell size of approximately 10 μm in diameter meaning that conventional multiwell plates are unnecessarily large for the study of individual cells. This fact together with the need for higher throughput, improved control of microenvironments, detailed study of cellular behavior, decreased amounts of reagent-use etc, favors miniaturized systems compared to conventional 96/384-well plates for single-cell analyses. Many of the previously presented systems allow for short-term (hours or days) analysis only (Deutsch et al., 2006b), while for a more complex analysis there is a need for longer cultivation times. The ability to study clone formation and to individually treat the different wells/compartment are highly beneficial to diagnosing leukemia. Furthermore, instead of using limited dilution for cell seeding, i.e. to randomly position single cells onto cell-chips, a way of controlled cell seeding increases throughput and controllability.
This study presents the development of a microwell plate designed to match a standard flow-cytometric cell sorter for controlled single-cell seeding into the wells of the plate, followed by clonal growth and detection (Figure 10).

Figure 10. Computer rendered animations of the procedure of drawing blood from a patient, individually sorting cells into microwells using FACS followed by culturing the cells for a number of days resulting in heterogeneous clone formation.

The microwell plate consists of three layers; a bottom glass plate (h: 175-500 μm), a silicon grid (h: 500 μm) anodically bonded to the glass plate, and a reversibly sealable PDMS top membrane (h: 500 μm) for sealing the individual wells during cell culturing. Microtiter plate formats (128 x 86 mm² with 3243 wells) or chip-formats (76 x 26 mm² with 672 wells) as well as a thin and flat plate offer compatibility to standard instrumentation such as microscopes. The number of microwells on the total surface was maximized, with the only limitation being the precision of the FACS, requiring a minimum of 1500 μm center-to-center distance between the wells (Figure 11a-c). Sloped wall-openings facilitated cell seeding into the 500 nl-wells.

Single cells from the leukemic cell line K-562 were seeded into the wells of the microwell plate with a success rate, i.e. the percentage of wells holding a single cell after FACS seeding (Figure 11f-g), above 90%. A strength of this method as compared to other single-cell microdevices is the ability of flow cytometric (FC) sorting to link cultivation data to data from the FC analysis by monitoring adherent or non-adherent cells as they expand into large clones during several weeks of proliferation (Figure 12).
Microwell devices for single-cell analyses

Figure 11. Photo- and micrographs of different well designs (a-e) and results from FACS sorting (f-g). (A): Version with 1536-6144 wells per plate, top left area shown in all images but d. (B): The structure of a well (red). (C): Distance between two wells (green). (D): Twelve “100×100 μm² wells” (white dots) with spacing (black) in between. (E): Four “650×650 μm² wells” (grey) - the standard format used for FACS sorting (and same as in b,c,g,f). (F): The total bottom area of a well with a fluorescent single cell successfully seeded by FACS. (G): One well, with a fluorescent single cell that has missed the well and sits on the sloped wall. Scale bars 500 μm.

An asset of the small well format was the ability to analyze the formed clones at an early stage (after two, three days) since they can be tracked and visualized (Figure 12a) from the first hour, as compared to standard multiwell plates where larger clones must be obtained (due to the dissimilarity between the well- and cell size), resulting in longer assay-times. Viability and the number of cells giving rise to clones and the various sizes of the different clones etc. were investigated in a time-dependent manner, exemplifying parameters suitable for analysis in the microwell plate. The natural goal from a clinical perspective is long-term clonogenic assays of primary leukemic cells, and in a pilot study the proliferation of primary cells was successfully detected in a small fraction of the wells. In clinical investigations drug responses on clones are interesting, wherefore drug distribution were performed and subsequent cell response investigated to prove the concept.
Figure 12. Micrographs of non-adherent (A) and adherent (B,C) clonal cell growth in the microwells. (A): Leukemia cells (K-562) at 0 (entire well shown), 5 and 20 days (close ups of colonies). (B): Osteosarcoma cells (U-2 OS) and (C): epidermoid carcinoma cells (A-431) after 7 days of microwell culture. Scale bars 100 µm.

In conclusion, a microwell plate for single-cell analysis and long-term cultivation was developed. A controlled number of adherent or non-adherent cells such as single cells, two cells etc. can be seeded into the wells of the plate using FACS seeding. The optical properties provided by the transparent glass bottom plate and opaque silicon grid, make the plate ideal for cell imaging. The well size is small enough to suit for single cells but large enough to hold large clones after several weeks of cultivation. The method should be useful in a range of cell-based assays requiring many samples to be studied in parallel, for example clonogenic assays to investigate drug response.
II. Culture and analysis of stem cells.

In this study, a high-density microwell chip was used as a screening device for stem cell research. Standard culture conditions, assays and protocols for downstream analysis such as marker screening were verified on chip for mouse and human embryonic stem cells and mouse adult neural stem cells.

Stem cells hold great potential in biological research and are characterized by multipotency and the ability to self-renew. Mammalian embryonic and adult neural stem cells are cultured and analyzed in vitro in a number of assays, including adherent growth on (pre-coated) surfaces or growth in free-floating sphere-like clusters, i.e. neurospheres (NS). By utilizing certain growth factors the pluri- and multipotency in different stem cell populations can be maintained. New factors are constantly being connected to stem cells and extensive studies focus on finding specific combinations of factors to direct differentiation, or reprogram mature stem cells into induced-pluripotent cells (Shi et al., 2008). Such screens require many samples to be run in parallel and due to the generally high cost of factor molecules, low-volume assays are beneficial from a cost perspective (Singec et al., 2006). Furthermore, long-term clone formation of stem/progenitor cells is highly interesting why tools for day- or weeklong analyses are sought after.

Figure 13. (A): Photograph of the chip. (B): Drawing of the sandwich design where the removable top membrane is added for cell culturing. (C): Photograph of an etched well number on top of the 140 μm wall (white).
The microwell chip used in this study is similar to the chip described in Paper I, the only modifications being a thinner glass bottom plate (h: 175 μm), a thinner cell culture membrane (h: 200 μm), and individually labeled wells (row and column position) resulting in improved imaging (Figure 13).

Standard handling of stem cells involve maintaining cells in a pluripotent state or analyzing cells that undergo differentiation. Stem cell maintenance requires daily change of growth medium why user-friendly protocols for on-chip handling was demonstrated. For non-clonal experiments the cells were seeded in a random manner, using a standard pipette seeding many wells simultaneously. Mouse ES cells, mouse adult forebrain neural stem cells, and human ES cells were verified pluripotent by fixation and labeling with the pluripotency markers Sox2 and Oct4, and counterstaining with the nuclear stain DAPI three days after cell seeding on chip. Furthermore, the commonly used NS-forming assay to demonstrate self-renewal was used on chip, resulting in sphere-formation of mouse adult neural cells after four days of culture. By seeding a single cell into each well, problems with movement-induced aggregation and uncertainty whether a sphere is clonally derived or not can be avoided. Examples of the verified pluripotent state of the different cell types are shown in Figure 14.

The microwell chip was also shown useful in differentiation studies of neural stem cells, where the chip was pre-coated with cell-specific extracellular matrix before seeding the cells. Cell differentiation was then carried out using growth medium containing factor molecules for directed differentiation. The three cell types were maintained on chip under differentiation conditions for nine days followed by verification of immunoreactivity to the neuronal marker βIII-tubulin and counterstaining with DAPI (Figure 15).
Figure 15. Micrographs of neural differentiation of (A): mouse ES cells, (B): mouse adult neural stem cells, and (C): human ES cells after 9 days of microwell culture. DAPI (blue), βIII-tubulin (red/green). Scale bars 25 μm.

In conclusion, a screening tool for stem cell culture and morphology analysis was demonstrated for maintenance and differentiation studies of stem cells. The small format showed suitable for non-fluorescent and fluorescent analysis and the large number of wells should enable hundreds of thousands of substances to be screened in a remarkably short time (days), verified by compatibility with a range of market-leading detection and liquid-handling instruments. The microwell chip should thus simplify work-intensive and costly screening experiments in the field of stem cell analysis, e.g. the study of cell differentiation, cell morphology, cell heterogeneity and effects of reprogramming factors on cells.
III. Imaging of a bispecific binding protein.

In this study, a bispecific (bs) affibody molecule for the simultaneous binding of HER2 and EGFR was engineered and characterized. Verification of successful targeting, was partly performed by immunofluorescence microscopy using a microwell chip. The small well-volumes of the chip were suitable for the study of the costly HER2-reagent and its binding of the bs affibody molecule.

An increasing number of clinically approved treatments for cancer are based on knowledge of molecular targets that are specific to tumors. Monoclonal antibodies (mAbs) are widely used for targeting of tumor-associated antigens, in \textit{in vivo} diagnostics as well as in therapy. A promising approach to improve selectivity, imaging contrast and potentially also therapeutic efficacy, are the simultaneous targeting of two different receptors as compared to the traditional one-target approach. For example, a study of breast-cancer patients shows a more severe prognosis for the patients with co-expression of EGFR and HER2, compared to those with expression of either EGFR or HER2 (Osaki et al., 1992). Thus, simultaneous targeting of EGFR and HER2 should be of value in molecular imaging efforts and could improve the selectivity for targeted therapy. Affibody molecules (Figure 16a) are alternatives to mAbs. They were developed by combinatorial protein engineering of the 58-amino-acid residue Z domain scaffold from staphylococcal protein A (Nilsson et al., 1987). Previously, affibody molecules targeting either HER2 (Orlova et al., 2006) or EGFR (Friedman et al., 2008) has shown high affinity. Therefore linking them together for dual (bispecific) receptor targeting was investigated in this study.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{affibody.png}
\caption{Schematic illustration of (A): an affibody molecule, courtesy of (Friedman and Stahl, 2009) and (B): a bispecific affibody molecule for dual targeting in which each affibody moiety consists of a triple $\alpha$-helical bundle. Below is the bs affibody construct including a N-terminal His$_6$ tag and a (G4S)$_3$ linker. (C): Micrograph of an EGFR-expressing cell (A-431) in the microwell chip visualizing nuclei (blue), cytoskeleton (green) and ER (red). The image was obtained by deconvolution of a z-stack of fluorescent images, courtesy of Leica Microsystems. Scale bar 10 $\mu$m.}
\end{figure}
A gene construct encoding a bs affibody molecule, targeting both HER2 and EGFR, was constructed by introducing a 20-amino-acid linker between the dimeric versions of the HER2- and EGFR-binding affibody molecules (Figure 16b). Binding was confirmed to immobilized proteins and to HER2- and EGFR-overexpressing cells in biosensor analysis and flow cytometric analysis, respectively. The next step was to study whether the bs affibody molecule could bind a cellular receptor with one “arm” while binding a soluble receptor with the other “arm”. Owing to the high reagent cost of soluble target protein, a micowell array holding 0.5 μl/well (Paper I) was used for microscopy-based determination of binding properties. The microwells had previously been shown to be suitable for high-resolution imaging (Figure 16c). Furthermore it offers many individual experiments to be run simultaneously (672 wells in total) which was convenient in this study (Figure 17a).

EGFR-expressing cells (A-431) were cultured in the microwells followed by incubation with bs affibody molecule and soluble HER2-ECD target protein and subsequently detected with an anti-HER2 antibody and fluorophore-conjugated secondary antibody (Figure 17b). Figure 17c demonstrates the successful dual binding of the bs affibody molecule to cells and soluble target protein compared to a negative control affibody molecule (Figure 17d). Finally, the successful simultaneous binding of
the bs affibody molecule to both receptors in their native forms, i.e. on cells, was demonstrated using real-time cell-cell interaction analysis.

In conclusion, the first reported bs affinity protein for simultaneous targeting of HER2 and EGFR was characterized. Potential use for the construct includes imaging and therapy applications with increased efficacy and selectivity. As a minor part of the study, a microwell chip was shown to be useful for high-resolution imaging utilizing the ability to run many experiments in parallel, conveniently collected on a well-array slide.
IV. Genetic analysis of clones.

In this study, a method for genetic analysis by PCR and minisequencing of cells cultured on chip was developed. Two human adherent cell lines: one wild type and one with a single-base mutation in the p53 gene were expanded into colonies in wells before in-well DNA analysis for mutation screening.

In cancer research, single-cell PCR is today the golden standard for studying heterozygosity. However, it can be problematic to rely on one single cell alone, since the well-known phenomenon of allele drop-out may lead to misinterpretation of the genetic status of the investigated cell (Findlay et al., 1995). Aiming for more robust methods for analysis of genetic variations, tens or hundreds of cells are commonly used as starting material (Pettersson et al., 2006). However, studying the detailed genetic information available on the single-cell level can be advantageous (even crucial for some applications). Hence, allowing a single cell to proliferate, yielding 10-20 clonal cells in a microwell, is an appealing methodology in order to provide sufficient material for robust PCR analysis. Microwells also provide the ability for many samples to be run in parallel, on a single chip. One example from sequencing demonstrating the usefulness of high-density platforms is the Roche/454 system, one of the large-scale DNA sequencing methods developed in order to reduce time and cost using picoliter sized wells (Margulies et al., 2005).

This work was recently highlighted in “Chemical Biology” by RSC Publishing. The effort was explained as an integrated system combining several established techniques: “Clones are grown in parallel from single cells held in microscale wells within a chip. The cells are then broken open to release their DNA and a polymerase chain reaction (PCR) is used to multiply the amount of DNA. This double stranded DNA is then captured onto magnetic beads and denatured to form single stranded DNA. Finally, a reporter molecule that fluoresces when it binds a particular DNA sequence is added, allowing mutations or differences between samples to be spotted. The researchers were able to use the system to distinguish cells from two human cell lines differing by one base mutation in their DNA” as cited from the text (Johnson, 2009) and schematically shown in Figure 18.
Figure 18. Overview of the method. Single cells are seeded in microwells (1) and cultured, forming clones (2). Subsequently, cells are thermally lysed and PCR reactions are performed (3). Since the primer pair consists of one biotinylated and one fluorescent primer the PCR products can be detected when captured by streptavidin-coated magnetic beads (4). After denaturation with NaOH (5) a minisequencing reaction can be performed (6) and the products can be detected using an array scanner (7).

The method thus facilitates linking of cell culture data, i.e. proliferation properties of individual cells, to genetic heterogeneity. The chip from Paper I was modified with a thicker glass bottom (1000 µm to enable detection with an array scanner) and a plastic film during amplification reactions to prevent evaporation. Many possible analytical options are offered using this method, for example direct detection of PCR products in the microwells (Figure 18:3) with no need for removing uncaptured primer due to the high local concentration of fluorophor on the beads (Figure 19).

This can also be utilized in allele-specific PCR in the microwells, where direct read-out from the sealed individual reaction chambers may be achieved. Further development of this methodology could enable solid-phase PCR to be run directly on the magnetic beads, subsequently detecting the PCR products immediately following the reaction without removing the plastic sealer, thus eliminating the need to add beads (i.e. open each well) before detection of the PCR products. The use of a bead-bound product (as in this study) conveniently enables washing steps throughout the protocol by maintaining the DNA strands in their respective well (streptavidin-labeled DNA strands on biotinylated magnetic beads) by the use of an underlying magnet.
Microwell devices for single-cell analyses

Figure 19. Micrographs of FITC (green) labeled PCR products detected captured on beads with no need to wash away remaining fluorescent primers in solution. The fluorescent channels (left) and the bright field channels (right) are shown. (A): Washed PCR products and (B): un-washed PCR products. Scale bars 20 μm.

The stepwise approach described here, to capture the PCR products on beads and denature the DNA, opens up for many applications including single stranded DNA analysis, exemplified by a mutation frequency analysis to distinguish mutated cells from wild type, using minisequencing (Figure 20).

Figure 20. Images from an array scanner where the targeted exon 8 sequence of the p53 gene is detected in (A): mutated A-431 cells by a Cy5 (red) signal, and (B): in wild type U-2 OS cells by a Cy3 (green) signal. Signals from both channels (Cy3,5) are shown simultaneously. Scale bar 300 μm.

In conclusion, by expanding single cells into clonal populations, lysing the daughter cells, amplifying the genetic material, performing a mutation analysis using minisequencing and detecting the products in all wells simultaneously using an array scanner, the system described provides a valuable tool for mutation analysis screening.
V. Integrated liquid handling.

In this study, microfluidic components were integrated with a microwell array to enable controlled liquid handling during single-cell culture and clonal analysis. Cell staining reagents were delivered to adherent cells cultured in the bottom of the wells by micropump-driven flow in microchannels on top of the wells to demonstrate the working principles of the system.

In pharmaceutical trials the interest to investigate cell response to different drugs, concentration gradients of drugs, and combinations of drugs, is high. Such studies combined with the opportunity to obtain information on cell heterogeneity from single-cell analysis could enable for example treating cancer cells individually to learn the particular combinations of drugs that only affect the diseased (target) cells. Existing single-cell tools generally treat all cells similarly with a shared reagent solution for all cells and wells (Di Carlo et al., 2006). This procedure requires careful, manual and generally laborious fluid handling with associated requirements for trained personnel and high risk of cross-contamination between individual wells. Liquid handling in the previously described microwell chip (Paper I) has been performed manually by rinsing the entire chip with medium or reagents (Paper II, IV) or by directed addition of reagents to a few wells only (Paper III). Since the design of the wells enable individual treatment of each well, an alternative solution for adding and changing liquids was needed.

Figure 21. Photographs of a PDMS channel on top of a row of wells is shown (A): from above and (B): from below. (C): External syringe pumps or similar instruments can be utilized to control the flow. Scale bars 3 mm.
Channels along the array were fabricated in PDMS to cover a row of wells when placed on top of the microwell array. The adhesive properties of PDMS (used in Paper I-IV for sealing individual wells during cell cultivation) seals each individual row of wells, facilitating liquid addition in a controlled manner. The microwell array together with a channel layer thus shape the simplest form of a microfluidic system, were for example an external pump can drive fluid through the channels (Figure 21). A more sophisticated system was developed in this study, where a micropump in the channel layer drives the flow, glass layers clamp the system together in sandwich fashion, and a PDMS reservoir layer enables storage of liquid and/or connection to external liquid handling systems if necessary.

To use the system independently, micropumps were constructed in the channel layer for generating flow through the channel without externally connected tubings (Figure 22). The pumps consist of cross-shaped rotors that are actuated by an externally rotating permanent magnet.

Flow simulations and experimental verifications of reagent delivery to the bottom of the wells resulted in successful staining of cultured adherent cells (Figure 23). Approximations of flow-induced stresses on the cells indicate that for example shear stresses are small enough to be neglected. Though, from a cell-biomechanical point of view it may be of interest to study shear forces and their effect on cells. For such studies, the system could be modified, changing the flow rate, depth and width of the channels. Additionally an external pump for achieving high enough flow rates may be applied.

The control of many parallel channels on a high-density single-cell chip can be advantageous in various screening applications. By interconnecting the different channels via valves and junctions, it is for example possible to merge two reagents and
study cell response dynamically. Since reagents can be applied during monitoring, rapid cell responses can be tracked. Furthermore, as compared to closed microwells the fluidic system connects a row of wells enabling the study of down-stream effects of cell signaling etc.

Figure 23. (A): Micrograph of adherent osteosarcoma cells (U-2 OS) stained with live/dead cell-stain. (B): Close ups on live cells (green) and (C): dead cells (blue). Scale bars 50 μm.

In conclusion, a fluidic microwell system was developed to enable parallel and controlled liquid handling for large numbers of individual cells. No external tubing and/or connections were necessary for generating flow but instead internal micropumps (driven by externally applied magnetic fields) controlled the fluid delivery to cells along the channels. Directed reagent supply to the individual rows of wells enables different cell treatments in parallel row of cells. This approach yields additional sets of data, compared to manual treatment of an entire chip with the same reagent.
6th chapter: Concluding remarks and outlook.

This thesis presents work on microwell devices that are all based on the same general chip design, i.e. a microwell plate with several hundreds of 500 nl-wells. The main conclusion is that a miniaturized platform allowing many parallel experiments in a standardized format, compatible with standard instrumentation, using low volume samples is highly beneficial for a wide range of single-cell applications.

A microwell plate (the terms chip, array, device are used synonymously) with an accompanying gas-permeable membrane were developed for highly parallel cell cultivation (Paper I). The first study presents growth of single non-adherent cancer cells in the plate for up to three weeks, without change of culture medium. Clone formation ratio, size and abundance are studied. The analyses of adherent cells (requiring pre-coating of the surface) is also demonstrated and validated. Single cells are positioned in the microwells in a controlled manner, using a flow cytometer with sorting ability. This methodology offers an array of compartmentalized single cells for instant or long-term analysis useful for example in drug efficacy studies. Clonogenic studies of leukemic primary cells utilizing this format may be helpful for screening-investigations of which drug (or combinations thereof) individual patients will respond to. Consequently the microwell plate is a tool with great potential for use in the progress towards personalized healthcare.

The platform has also shown usefulness in screening attempts related to stem cell research (Paper II). Stem cells from human and mouse can be maintained pluripotent, and investigated for neural differentiation in the microwell chip. A protocol for user-friendly practical handling of the chip is described, enabling an efficient means of analyzing stem cells in a miniaturized device. The chip offers a drastic time-reduction for large-scale factor molecule screening, an increasingly interesting area for example in investigating the role of stem cell reprogramming factors.

Low volume microwell chips may save costs in experiments involving high-cost reagents, utilized in the study of a bispecific affinity protein targeting the two cancer-associated receptors HER2 and EGFR (Paper III). The chip is used as an immunofluorescence microscopy slide for high-resolution imaging of protein binding.
As such it also allows performing many parallel experiments, for example combinations of primary and secondary antibodies for cellular protein-detection.

Cell and protein studies in microwell chips are also interesting from a genetic perspective, why PCR compatibility in the microwells was investigated (Paper IV). Cultured clones are lysed and subjected to PCR directly in the microwells. Utilizing a biotinylated primer and a fluorescently labeled primer, the products are captured on streptavidin-coated magnetic beads and detected in a fluorescence microscope. Furthermore the double-stranded DNA is denatured and washed while maintaining the strands immobilized in the microwells by applying a magnetic field. This opens up for mutation analysis, using sequence-specific detection where mutated cells are distinguished from wild type cells rapidly using a conventional array scanner. Screening genes from clones is a potentially powerful alternative to single-cell PCR, in terms of robustness and sensitivity.

The fact that the microwell chip consists of silicon and glass means that it is compatible with many microfabrication techniques. For improved control of addition and removal of liquid to specific wells, the cell culture membrane was modified to add channels for directed flow (Paper V). An external rotating magnet controls the microrotor in the channel layer, yielding an independent micropump that can be used for delivering reagents to adherent cells without the need for external pumps or tubing. Integration of liquid handling on the chip thus enables controlled addition of liquids to chosen wells, and by connecting many wells downstream effects such as cell signaling can be studied. The system should also be useful in drug-response studies where gradients of drugs can be added to cultured clones for dynamic studies of living cells at high throughput.

In the process of developing the microwell plate, compatibility with standard instrumentations was considered as important, exemplified by the use of FACS (Paper I,II), microscopes (Paper I-V), an array scanner (Paper IV), thermocycling instruments (Paper IV) and pump integration (Paper V). Internal design (e.g. center-center distances of wells for cell seeding by FACS in Paper I) and external design (e.g. the outer format and glass thickness of the chip for matching a conventional array scanning instrument in Paper IV) are both important. User-friendliness was emphasized for the stepwise approach to practical use and adaptation by biologists (Paper II). Flexibility was considered important throughout the studies as exemplified
Microwell devices for single-cell analyses

by the many possible choices of top layer in the chip/sandwich design; PDMS membrane for long-term cell culture (Paper I-V), cover slip for instant analysis (Paper II-IV), plastic film for high-temperature and evaporation-critical assays (Paper IV), and integration of channels for controlled liquid handling (Paper V). Disposability vs. reusability is interesting and as impurities have shown to accumulate in the wells over time, the chip can only be reused for certain applications.

The outlook for these particular microwell devices is promising due to their great adaptability to various biological assays. Two main future directions for these devices are introduction of new applications or the use of an existing application and employ it for gaining a deeper biological understanding. The second alternative is, from an end-perspective, more interesting due to the great potential of this device to actually reach the clinics and to add value to cell-based assays at hospitals and laboratories. What the final “killer-application” might be is too early to tell, but clonogenic studies and drug effects or molecule screening in combination with cellular heterogeneity studies are all examples of assays that benefit from this and similar systems. Advantages of the microwell chip are its simple configuration and its similarity to conventional 96-well plates. Since biologists are used to multiwell plates, the step to adapting the microwell version should not be perceived as a long or difficult one to take. For practical use, automated instruments (e.g. dispensing robotics and detection systems) working with small volumes needs to be integrated with the microwell plate. Such instruments already exist and since all experimental platforms in life-science aim for higher throughput, it is only a matter of time before nanoliter instruments are standard equipment in laboratories and miniaturized devices are commonly applied.

Yet, the small wells are “frightening many” and the practical handling of them seems “complicated” from a biologist’s perspective. In general, a single-cell analysis tool must offer significant advantages and a great added value before biologists are convinced of their usefulness. If the same analysis can be made with conventional methods, biologists will prefer this (since they normally are not technologically interested and do not value a new method per se). The added value of this and similar methodologies lies in enabling biological discovery by means previously not thought possible. In that perspective, single-cell and heterogeneity-studies have high potential. The key to success is to reveal areas where conventional (macro-) solutions perform poorly, and turn that insight to targets of opportunity for microdevices. Laser capture
microdissection is an illustrative example; cells are grown or placed on a heat-sensitive surface before they are cut with a focused, pulsed, ultraviolet laser. Although the protocol might seem strange for a first-time user, it has been widely adapted due to the benefits of the method and the added value shown in terms of efficient collection and analysis of small numbers of cells.

One future application of the platform discussed in this thesis could be in vitro fertilization (IVF). The microwell plate should provide detailed control of the ova and the sperm in a highly parallel and timesaving fashion. The small wells could maintain individual sperm cells within the microscopic field of view in order to study motility, morphology etc. of the cells over time. Furthermore, micromanipulators could be utilized for accessing the individual microwells, providing a complementary high-throughput IVF platform. Similarly, the interaction of immune cells and their target cells could be studied in detail using this and similar platforms. To conclude, the microwell platform discussed in this thesis should have high potential in various biological assays where the parallel, low-volume and long-term analysis of individual cells is desired.
Grunden för mikro- och nanoteknik är att förminska utrustning och tekniker samt att utnyttja de fenomen som endast existerar i liten skala. En nanometer är en miljarddels meter och för att kunna studera molekyler som själva är i den storleksklassen, använder nanotekniken verktyg/provrör i samma storleksordning.


Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>LOC</td>
<td>Lab on a chip</td>
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<tr>
<td>μTAS</td>
<td>Micro total analysis system</td>
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<tr>
<td>MEMS</td>
<td>Microelectromechanical systems</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>mAb(s)</td>
<td>Monoclonal antibody (-ies)</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>AM</td>
<td>Automated microscopy</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>FC</td>
<td>Flow cytometry</td>
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<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
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<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
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<tr>
<td>LSC</td>
<td>Laser scanning cytometry</td>
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<tr>
<td>NS</td>
<td>Neurosphere</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem (cell)</td>
</tr>
<tr>
<td>Sox2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>Oct4</td>
<td>Octamer-4</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>Class III beta tubulin</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal growth factor Receptor 2</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>bs</td>
<td>Bispecific</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
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<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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Epilogue.

The last years have been fantastic and I have really enjoyed being a PhD student. I have learnt a lot and am now looking forward to the near future. Many people have been involved in my work toward this thesis and I wish to thank everybody that has contributed in one way or another. Some of the people are briefly acknowledged below.

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Sara Lindström


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