Single Cell Investigations of the Functional Heterogeneity Within Immune Cell Populations – a Microchip-based Study

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To my family
Abstract

Immune cell populations are constantly divided into smaller and smaller subsets defined by newly emerging cellular markers. However, there is a growing awareness of the functional heterogeneities in between cells even within small populations, in addition to the heterogeneity over time. One may ask whether a population is correctly defined only by cellular markers or if the functionality should be regarded as well? Many of today’s techniques only measure at the population level, giving an average estimate of the behavior of that pool of cells, but failing to detect rare possibly important events. Thus, high-throughput experimental approaches to analyze single cells over time are required to address cellular heterogeneity.

Progress in the fields of microfabrication, microscopy and computing have paved the way for increasingly efficient tools for studies on the single cell level, and a variety of devices have been described by others. However, few of them are suitable for long-term imaging of dynamic events such as cell-cell interactions or migration. In addition, for efficient recording of many individual events it is desirable to scale down the cells’ interaction volume; not only to shorten the time to interaction, but also to increase the number of individual events in a given area; thereby pushing a screening approach.

To address these questions, a complete microwell array system for imaging of immune cell responses with single-cell resolution was designed. The platform consists of a range of silicon-glass microchips with arrays of miniature wells for incubation of cells and a custom made holder that fits conventional microscopes. The device has been designed to allow cells to be kept viable for several days in the wells, to be easy to use and to allow high-resolution imaging. Five different designs were fabricated; all with a specific type of assay in mind, and were evaluated regarding biocompatibility and functionality. Here, the design aimed for screening applications is the main focus. In this approach a large amount, tens of thousands, of small wells are imaged two to three times: first directly post-seeding of effector and target cells to register the well’s content, and second after some time has passed to allow for cell-cell interactions. The final read-out is the number of killed target cells in each well, making an automatic cell counting protocol necessary in order to analyze the massive amount of data generated.

We here show that our silicon microwell platform allows long-term studies with the possibility of both time-lapse and high-resolution imaging of a variety of immune cell behavior. Using both time-lapse imaging and the screening approach we confirmed and investigated immune cell heterogeneity within NK cell populations in regards to both cytotoxicity and migrational behavior. In addition, two different types of cytolytic behavior in NK cells, termed fast and slow killing, were described and evaluated in regards to dynamic parameters; like conjugation and attachment time. We could also quantify the type of cytolytic response in relation to serial killing NK cells, and saw that serial killing NK cells more often induced fast target cell death. Further investigations using the screening approach have shown that serial killing NK cells also differ from other NK cells in their morphology, being both larger and with a
more elongated shape. So far the platform has been used to gain better understanding of some aspects of NK cell biology, but there is still much left to explore. With the addition of an automatic counting program, the large numbers of wells that can be simultaneously imaged will provide new statistical information and enable higher throughput. 
Altogether, our family of techniques enables novel types of cellular imaging assays allowing data collection at a level of resolution not previously obtained – this was shown to be important for performing basic cell biological studies, but may also prove valuable in the proposed future medical applications such as adoptive cell therapy and stem cell transplantation.
List of Publications


Related papers not included in the thesis:

Contribution by the author

Paper I: I was involved in the design and development of the method, performed and analyzed all the biological experiments. I wrote the main part of the paper.

Paper II: I was involved in the design and development of the method, and performed and analyzed a majority of the biological experiments. I was actively involved in the writing process and designing figures.

Paper III: I performed part of the biological experiments and the analysis of those. I was also actively involved in the writing process and design of figures for publication.

Paper IV: I partook in some of the biological experiments, but was mainly involved in the data analysis of. I was also involved in the writing process.

Paper V: I was involved in development of the method. I performed and analyzed most of the experiments. I was also involved in the writing process and figure design.
Populärvetenskaplig sammanfattning


Alla kroppens celler har en mängd olika proteiner och andra ämnen på ytan som signalerar om cellens status, och en del av dessa kan antingen upp- eller nedregleras vid till exempel infektioner. Till exempel presenterar all na celler konstant små sönderklipppta bitar av alla proteiner de innehåller med hjälp av ett specialiserat ytprotein som kallas major histocompatibility complex (MHC). Om cellen blir infekterad med ett virus kommer den således även att presentera proteinbitar från viruset! Dessa kroppsfrämmande proteinbitar, så kallade antigen, kan då kännas igen av immunförsvaret som dödar den infekterade cellen.


Natural Killer (NK) celler är en del av det nativa immunförsvaret som därför utbildas i att känna igen celler som saknar viss typ av MHC på ytan. NK celler patrullerar blodet och lymfan och undersöker cellerna i sin omgivning genom att bilda en så kallad immunologisk synaps – en tät inter-cellulär kontakt där proteiner från NK cellen och den undersökta cellen kan mötas. Beroende på mängden aktiverande och inhiberande signaler avgör den huruvida den ska döda cellen eller inte. Även många tumörceller förlorar MHC, vilket gör att NK-celler även är en viktig del av kroppens skydd mot cancer.

för patienten. Att bättre kunna förutspå risken för detta vore på så vis oerhört värdefullt.


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1 Introduction

1.1 The Immune System
Our body is constantly being exposed to various infectious pathogens and other harmful substances present in our environment. The first line of defense is the physical and chemical barriers preventing access to our bodies; for example the skin and acidity of the gut; but this is generally not considered part of the immune system. Instead the immune system is comprised of specialized effector cells and molecules acting together to protect us from being infected or otherwise harmed. The main threats are microbes such as bacteria, viruses and parasites, all depending on the shelter and nutrient supply a human body can provide for their own survival. But the immune system do not just protect us from external threats, it can also act to clear self cells that are potentially harmful, e.g. cancerous cells.

1.1.1 Adaptive and Innate Immunity
There are two types of immune responses to an immunological challenge, both depending upon the activities of leukocytes. The innate immune system starts working fast and helps control the infection while the slower but more efficient adaptive immune response develops. The initial response to an infection is usually inflammation caused by leukocytes of the innate immune system, so called because it is more or less present in the same form at birth throughout life. Innate immune cells recognizes certain well conserved patterns on the pathogens, and can in response eliminate them by phagocytosis as well as release signal molecules called cytokines that cause inflammation and alert nearby cells of the danger. Cytokines also help attract more immune cells to the site of infection, enhancing the immune response and possibly clear the infection. However, many pathogens have evolved strategies to evade the actions of the innate immune system and can only be cleared by the adaptive immune system.

The adaptive immune system is educated throughout life in every challenge with a new pathogen. This immune response is much slower, taking days rather than hours to develop its full potential, but can very specifically recognize and eliminate specific pathogens. The adaptive immune system is comprised of T cells and B cells, eliciting their functions via surface receptors that are specific and unique to each cell. These cells undergo a unique process in which their DNA at a particular location is cut up and scrambled to generate a receptor that is completely unique, and can be almost infinitely diverse. As a result, T-cell receptors (TCR) and B-cell receptors (BCR) are capable of recognizing just about anything, because each individual cell has a unique receptor that is incredibly specific. To prevent autoreactivity, cells with receptors
recognizing self-peptides undergo apoptosis in a process called negative selection. A unique feature of the adaptive immune system is its capability to generate immunological memory. After the infection is cleared some adaptive immune cells can turn into specific memory cells, these cells can then on a subsequent challenge with the same pathogen be activated and elicit an immediate very specific reaction. Often the infection is then cleared without the host even noticing. This is the reason why some infections are only experienced once, and it is also the mechanism behind successful vaccination.

1.1.2 Natural killer cells
Natural killer (NK) cells were first described in 1975 as lymphocytes with both cytotoxic and cytokine-producing effector functions. They are traditionally regarded as part of the innate immune system, as they depend on germline-encoded receptors and do not undergo a receptor gene rearrangement in response to pathogen stimuli. They have been officially classified as members of the group 1 innate lymphoid cells (ILCs), which are defined by their capacity to secrete interferon (IFN)-γ but not type 2 cytokines. During early innate immune response they influence both the recruitment and function of other hematopoietic cells, e.g. other cytolytic cells such as T cells, and function in the regulatory crosstalk network with dendritic cells and neutrophils to either dampen or increase immune responses.

In addition, it has become increasingly clear that NK cells also show some features generally associated with adaptive immunity, such as a simplified form of immunological memory first described by Sun et.al. Recently, new evidence of 3 types of long-lived memory responses elicited by NK cells have been reported: 1) in a mouse-model virus-experienced NK cells survived for 70 days and readily proliferated upon re-challenge, and a similar phenomena has been observed in human transplant patients; 2) human NK cells in vitro prestimulated with a cytokine cocktail showed enhanced IFN-γ response to restimulation with the same cytokines up to three weeks later – indicating a sort of cytokine-mediated memory response; 3) identification of a liver-derived NK cell population in mice that generate antigen-specific memory responses to both haptens and viruses at least 4 months after the initial challenge. The memory-like responses described here are all less long-lived than for the adaptive immune cells, memory T and B cells can last for years, but these findings are still intriguing.

In humans NK cells are bone marrow-derived lymphocytes that comprise 5–15% of the peripheral blood lymphocytes. NK cells recognize foreign, tumor- and virus-infected cells and kill them by cytotoxic molecules stored in specialized secretory lysosomes called lytic granules. Recognition and killing of target cells is achieved by formation of an immune synapse (IS), a highly organized and dynamic sub-cellular interface, where activating and inhibiting receptors on the NK cell interacts with surface molecules on the target cell. The integrated signaling then potentially leads to downstream effector functions –
where responsiveness is thought to be determined by the strength of the inhibitory input received by the individual NK cell during education \(^{12,13,14}\). The IS was originally described in the late 1990s between T cells and antigen-presenting cells where T-cell receptors interact with major histocompatibility complex (MHC) molecules forming supra-molecular activation clusters (SMACS)\(^{15,16}\). Later a similar structure was described also for NK cells \(^{17}\).

In many viral infections MHC class I expression is downregulated to avoid detection by the adaptive immune system. NK cells recognize the lack of MHC class I expression on a potential target cell - and this recognition together with ligation of other activating receptors activates the NK cell. This is the basis for the ‘missing self’ hypothesis first proposed by Kärre et al. in 1986\(^{18}\). Normal expression of class I MHC antigens on the other hand, inhibits the cytotoxic action of NK cells \(^{19}\). However, the fate of a target cell is not solely dependent on the expression of MHC as it depends on delicate balance of many activating and inhibitory factors.

NK cells are able to kill their targets by at least two different mechanisms; slow killing by inducing apoptosis through death receptors and ligands, and rapid killing through degranulation of cytolitic compounds in close proximity of the target cell \(^{20}\). NK cells are not only cytotoxic but also have regulatory properties and can modulate the adaptive immunity via production of cytokines. Upon stimulation NK cells can rapidly produce e.g. IFN-γ, TNF-α, GM-CSF, IL-5, IL-10 and IL-13 \(^{21-23}\), thereby being able to exert both pro-inflammatory and immune regulatory roles.

### 1.1.2.1 NK cell subsets

In humans two major subsets of NK cells can be distinguished based on their expression levels of the cell surface proteins CD56 and CD16, namely CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) \(^{23}\), where CD56\(^{\text{bright}}\) cells have very low expression or completely lack CD16. The two sub-types differ in their maturation level, where the CD56\(^{\text{dim}}\) subset are fully mature and the predominante cytotoxic subset. The CD56\(^{\text{bright}}\) subset is mainly considered cytokine producers and constitutes approximately 5-15 % of the total NK cell population. They are also better adapted to leave the vasculature and are the main subtype of NK cells found in lymph nodes or the decidual tract of pregnant women, where they intriguingly make up approximately 70% of the lymphocytes during the first trimester of the human pregnancy. Recent evidence suggests that they play important roles in promoting angiogenesis during pregnancy \(^{24}\).

Over the years, the classification of NK cells into increasingly smaller subsets have been constantly carried out by the NK cell community, as new cell surface receptors are constantly being discovered or their function better understood. In the beginning NK cells were often referred to as ‘null’ because they were not thought to express any defining cell surface markers that could be used to distinguish them from other classes of leukocytes \(^{25}\). However, meanwhile elucidating their origin and relationship to other hematopoietic cells,
more and more markers, not just for NK cells in general, have been described. Today complex combinations of these are used to describe ever so small subsets. For example, a recent study revealed the existence of more than 6000-30 000 phenotypically distinct NK cell subsets in the blood of a single human being using the powerful tool of masscytometry. While it still remains to be seen whether it is feasible to make use of or even analyze such vast heterogeneity, one can still appreciate the meaning of evolving such diversity within the NK cell population – probably for a good reason in a world where the immune system is constantly challenged with pathogens and transformed or stressed host cells.

1.1.2.2 Inhibitory and activating NK receptors

As mentioned earlier NK cell activity is dependent on a delicate balance of activating and inhibitory input both from target cells and during education. It is now known that killer-cell immunoglobulin-like receptors (KIRs) are the predominate receptors for regulation of NK cell activation in humans. Following the postulation of ‘the missing-self’ hypothesis the search for the responsible MHC I receptors begun – and in the early 90’s they were first identified in mice (Ly49 receptors) and then in humans as KIRs.

KIRs come as both inhibitory and activating receptors, where the ones carrying a short cytoplasmic tail are generally activating and the ones with a long cytoplasmic tail generally inhibiting. There are 13 expressed KIR genes and the ligand is known for 7 of those. Inhibitory KIRs recognize mainly human leukocyte antigen (HLA)-C molecules – HLA is the name of the human MHC, and for which the isotype denoted by addition of a letter or combination of letters. HLA-A, -B, and -C are the major MHC I isotypes in humans. Another important HLA is the HLA-E because it has a specialized role in cell recognition by NK cells. It is a non-classical MHC molecule, instead of presenting a random foreign or self peptide it presents a signal peptide from MHC I molecules themselves, thereby constituting a second line of self-presentation. HLA-E itself is not recognized by KIRs, but instead by the inhibitory dimer CD94/NKG2A.

KIR genes are highly polymorphic and polygenic, giving raise to many human haplotypes, on top of this they also show a high variability in copy number. Because of this and their importance for NK cell activation, it is not surprising that variations of KIR/HLA interactions can affect human health. For example, there is a higher incidence of preeclampsia in pregnancies where there is a high affinity maternal KIR/fetal HLA-C interaction (strong inhibition is bad). There are other studies showing how different combinations of KIR/HLA can influence susceptibility of virus, as shown for hepatitis C and HIV-control. Naturally researchers and the medical community try to understand and explore these features to optimize and develop new treatment strategies based on manipulating NK cell function. Of particular interest is the increased understanding of how KIR/HLA matching/mismatching influence protocols used for HSC and adoptive NK cell transplantation. Up to now this strategy have
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proven most efficient when treating patients with acute myeloid leukemia (AML), but will probably also be tested in patients with other types of cancers \(^{38}\). More recently, an anti-KIR antibody (IPH2101) that blocks MHC-I recognition was shown to boost human NK cell function both \textit{in vitro}, in humanized mice\(^{39, 40}\), and in clinical trials in cancer patients \(^{41-43}\).

1.1.3 T cells

T cells develop in the bone marrow and travel to the thymus for maturation into naïve CD4\(^+\) or CD8\(^+\) T cells, recognizing MHC class II and class I respectively, and are subsequently released to circulate the lymphatic system. Here specialized antigen presenting cells (APCs), macrophages and dendritic cells, display foreign peptide fragments presented within the MHC complex. When the receptor on a circulating naïve T cell (together with either the CD8 or CD4 co-receptor) recognizes its specific antigen and binds to it the T cell can be activated, it then starts proliferating and can differentiate into one of several types of effector T lymphocytes.

The CD4\(^+\) cells are known as T-helper cells, they provide essential additional signals that activate antigen-stimulated B cells to differentiate and produce antibodies. CD8\(^+\) T cells or cytotoxic T lymphocytes (CTLs) kill cells that are infected with viruses or other intracellular pathogens. Because the surfaces of other virus infected cells display the same virus fragments in combination with Class I MHC markers, the activated CTL can quickly recognize, attack, and destroy the diseased cell thereby preventing virus replication. T cells are also implicated in transplant rejection.

1.1.4 Hematopoietic Stem Cell Transplantation and Graft Versus Host Disease

Hematopoietic stem cell transplantation (HSCT) is used primarily for hematologic and lymphoid cancers but can also be a potential treatment for other disorders. Transplantation of genetically non-identical bone marrow (allogenic transplantation) first became feasible in the early 1960s, after the identification and typing of human MHC complex (human leukocyte antigen (HLA)). The genes for HLA are closely linked on chromosome 6 and are inherited as haplotypes. Thus, two siblings have about one chance in four of being HLA identical. Allogenic grafts may initiate immune reactions related to histocompatibility in their new host if donor and recipient are not properly HLA-matched. The severity of the reaction depends on the degree of incompatibility, which is determined by the polymorphic class I and class II HLAs and the small peptide antigens from degraded proteins they bind.

Recipient T cells can recognize foreign donor antigens and thereby reject the new graft; this is why myeloablative and immunosuppressive regimes like total body irradiation (TBI) and/or chemical treatment is employed to suppress the recipient’s immune defense before transplantation. Donor lymphocytes can
recognize recipient antigens causing immune reactions against the recipient tissue; unwanted as in the potentially lethal inflammation called graft-versus-host-disease (GVHD), or beneficial as is the case when graft-versus-tumor effects help clear the cancer.

Chronic GVHD is the most serious and common long-term complication of allogeneic HSCT occurring in 30% to 70% of transplanted patients. The general treatment is prolonged immunosuppressive treatment, which increases the risk for serious infections and other complications. Because of higher treatment-related mortality, chronic GVHD remains the major cause of late death despite its association with a lower relapse rate.

Absolute prevention of GVHD is not possible, and it is always a risk when receiving a transplant from anyone else. Unfortunately it is not possible today to predict with certainty whether this condition is going to occur with any precision. Only small subsets of T cells are usually involved, but upon activation they proliferate and can pose a serious threat to the patient. This subset is not possible to detect with current experimental procedures.

1.2 Techniques

1.2.1 Optical Microscopy

Optical microscopy or light microscopy refers to the inspection of the sample at higher magnification. Fluorescent microscopy is a widely used method in biological research, and was used in a majority of the experiments in this thesis. To acquire additional data to the transmission bright-field image, one can sample information from one or more fluorescent channels. This requires that the objects of interest fluoresce which can be achieved with various labeling strategies. Fluorescence is the emission of light that occurs (often within nanoseconds) after the absorption of light that is typically of a shorter wavelength. An excited electron can take different routes via different energy states when returning to its ground state; this can be illustrated with a Jablonski diagram (Fig. 1). The difference between the exciting and emitting wavelengths is known as the Stokes shift. By filtering out the exciting light without blocking the emitted fluorescence, it is possible to see only the objects that are fluorescent.

1.2.1.1 Fluorescent labeling

Molecules that are used because of their fluorescent properties are called fluorophores. The wavelengths of absorption and emission, together with its fluorescent efficiency, are all determined by its lowest energy electrons – because those are easily excited. For imaging of biomaterial like living cells we use fluorescent probes, which combine the fluorescent properties of the fluorophore with the equally challenging task of molecular recognition. This makes it possible to use them in a fluorescent microscope to obtain clear
images of stained structures of interest. Fluorescent probes come in a plethora of variants, all optimized for different applications. Some are coupled to antibodies for staining of specific proteins; others will target specific cell compartments, like the nucleus, lysosomes or the cytoplasm. Fluorophores have also been developed to take advantage of the fact that a fluorophore’s absorption properties can be highly sensitive to a change in milieu. Fluorescent sensors can for example change their absorbance and/or emission spectra when bound to calcium ions, hydrogen ions or other molecules of interest. In addition, usage of intrinsically fluorescent gene products, green fluorescent protein (GFP) being the most famous, now allows molecular biologists to genetically tag protein components of living systems opening up for new possibilities in fluorescence based methods.

![Jablonski diagram](image)

**Figure 1. Jablonski diagram.** There are a number of possible routes by which an excited molecule can return to its ground state. A rapid return (I) via singlet states results in fluorescence and a delayed return via the long lived stable triplet state results in phosphorescence (II).

Many fluorescent probes used in this thesis belong to a group of cell-permeant dyes where the carboxylic acids have been modified with acetoxyethyl (AM) ester groups, resulting in uncharged molecules. Examples of these are the family of Calcein dyes. These dyes can freely diffuse over the cell membrane. Once inside the cell intracellular esterases hydrolyze the ester bonds reforming the carboxyl groups -the probe is polarized and leaks out of the cell much more slowly than it entered. In some cases the probe is even non-fluorescent until it is hydrolyzed. This family of probes gives a uniform fluorescent staining of the cell’s cytoplasm as long as the cell’s membrane remains intact. When a cell dies the membrane is no longer intact and the dyes leaks out, therefore these probes are often used for viability applications.
Another type of fluorescent probe binds to primary amines, which are present in proteins and other biomolecules on the inside and outside of cells. One example of this type of dye used in this thesis is DDAO. Another type of dye are the lipophilic dyes that do not pass through the cell membrane, but rather stain the lipid membrane itself. An example of this type is DiD. Both of these types of dyes will stay even after the cell is dead until the membrane is completely disintegrated.

1.2.1.2 Confocal microscopy

The confocal laser scanning microscope (CLSM) is an essential tool for many biomedical imaging applications. It is an optical imaging technique used to increase the optical resolution and contrast compared to conventional light microscopy. This is done by using point illumination of the sample combined with a spatial pinhole in front of the detector, eliminating all out-of-focus light outside the focal plane.

![Figure 1. The principle behind the epi-illuminated laser scanning confocal microscope. Rotating mirrors are inserted between the laser and the object to permit scanning of the object in three dimensions at high speed. Since the illuminating and fluorescent light both pass through the same lens and are reflected from the same scanner mirrors, only one pinhole is required.]
Excitation of the sample is realized by illumination with laser light passing through a dichroic mirror, i.e. a mirror that selectively reflects certain wavelengths while others are allowed to pass. The resulting emitted light has a longer wavelength than the exciting light and can thus be separated from unwanted reflected laser light, selectively sending the emitted signal towards the photomultiplier detector (Fig. 1).

The x-y scanner is comprised of a set of mirrors directing the laser light to one point of the specimen. Slightly tilting the mirrors in either x- or y- direction changes the angle of the laser and illuminates the next point on that axis. A full image can then be created by scanning over the whole specimen detecting one point at a time.

Thin optical slices of thick specimens can be made in the confocal microscope by only allowing light from the focal plane to reach the detector. This is performed with use of a pinhole aperture, which is placed so that light from in focus regions (whole line in Fig. 1) of the specimen is also in focus at the pinhole. Mostly this light can pass through the small pinhole (pinhole size is optimized for the emitted wavelength) and reach the detector, whereas light from other regions (dotted line in Fig. 1) will be blocked. By adding together several slices from different focus positions a high-resolution 3-D reconstruction of the specimen can then be made. A confocal microscope has a slightly better resolution horizontally (x-y) than vertically (z). The best horizontal resolution is approximately half the emitted wavelength; in practice about 0.2 µm, and the best vertical resolution is < 1 µm.

The CLSM has several applications, which include imaging of thin optical sections, multiple wavelength images, 3-D reconstruction of living cell and tissue sections. With an open pinhole the microscope may also be used as an ordinary fluorescent microscope, except that it scans the specimen.

1.2.2 Microfabrication methods

Microfabrication is the broad general term describing the processes of fabrication of miniature components and systems, of micrometer sizes and smaller, e.g. lab-on-a-chip devices. The technologies originate from the microelectronics industry, and the devices are usually made on silicon wafers even though glass, plastics and many other substrates are also used.

Two standard microfabrication methods were used for making of in the silicon microwells used this thesis, deep reactive ion etching (DRIE) and anodic bonding. Both of them are briefly described in this section. For a few applications the inverse structure of silicon microwells were made and used as masters for polydimethylsiloxane (PDMS)-molding of soft microwells. The making of these will be further discussed in the Material and Method section.
1.2.2.1 Deep reactive ion etching (DRIE)

Etching is the partial removal of a thin film or substrate using an etching agent, such as an acid or ion containing plasma, which chemically or physically attacks the substrate.

DRIE is a method for directed vertical etching, most often used for silicon. It is performed by alternating isotropic etch steps and passivation by deposition of a chemically inert layer. Isotropic etching has the same etch rate in all directions, compared to anisotropic etching which has different rates in different crystal plane directions.

Figure 2. DRIE of silicon. The first step is the photoresist patterning of the silicon wafer (I. – III.). A photoresist layer covering the silicon wafer is exposed to UV-light through a patterned glass mask, enabling removal of the exposed photoresist in a developer bath, resulting in a patterned wafer. The next step is the etching (IV.-VI.). The photoresist is inert to the etching agents used so only the exposed areas are affected. By alternating passivation and etching steps a continuously deeper pit is made.

The starting material is a standard p-type thin silicon wafer (300-500 µm thick) covered with a layer of photoresist, a photosensitive polymer solution. UV-light exposure of the wafer through a patterned chromium-glass mask removes the photoresist only in the exposed areas, transferring the pattern to the photoresist on the wafer. During the first etching step, only the areas that lack photoresist will be affected, resulting in a shallow pit. During the passivation step a chemically inert fluorocarbon layer, C₄F₈, is deposited all over the structure, protecting the entire substrate from further chemical attack thus preventing further etching. However, during the next etching phase, the directional ions that
bombard the substrate attack the passivation layer at all horizontal surfaces (but not along the sides).

Alternating these steps is repeated until the desired etch depth is achieved. The length of etch phase determines the shape of the well; the shorter the etch phase the smoother the walls, but longer etch phase will yield higher etching rate.

1.2.2.2 **Anodic bonding**
Anodic bonding is a method to permanently bond glass to silicon. The substrates are bonded at elevated temperature (~400 °C) by placing and clamping the substrates between two metal electrodes, and applying a strong electrical field (100-1000V) over the electrodes. At the elevated temperature, sodium ions in the glass are displaced from the bonding surface by the applied electrical field. Depletion of sodium ions near the glass surface makes it highly reactive with the silicon surface, thus forming a solid chemical bond holding the wafers together.

1.2.3 **Single cell technology**
Many of the conventional methods used in cell biology research only read out the average response of large populations. However, individual cells may respond differently to e.g. drug treatments or interactions with other cells, and by having experimental read-outs based on population averages, detection of rare clones or uncommon events become impossible. Lately it has become increasingly clear that most cell populations are very heterogeneous, and with that comes a renewed interest in analyzing cells on a single cell level. The ongoing development of e.g. microfluidic and computing tools constantly facilitate high-throughput analysis of cellular heterogeneity.

1.2.3.1 **Flow-based technologies**
Probably the most widely used method for single-cell analysis is flow cytometry, allowing 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. This method is widely used in immunology and sample throughput is continuously increasing, as is the number of parameters that can be scored simultaneously. Partly possible due to the increasing capacity of newer instruments, but equally important is the development of new dyes, for example tandem dyes and the possibility to ‘barcode’ samples (with fluorescent tags of varying brightness). Yet another advancement to the method is the commercialization of spectral flow cytometry, which collects and analyzes the complete fluorescence emission spectra from all fluorochromes at once. The spectrum is then deconvoluted to quantify individual fluorochromes, which in
theory makes it possible to distinguish more accurately between fluorochromes with highly overlapping emission spectra.

Mass cytometry is another newly introduced technology, where the number of simultaneously measured parameters increase substantially, sometimes up to 34 cellular parameters. This method overcomes the general limitation of spectral overlap present in most fluorescence-based methods by conjugating the detection antibodies to rare metals. (The problem of for example auto-fluorescence is also eliminated because metals do not exist in hematopoietic cells normally). Metals also have a unique mass, which makes compensation unnecessary since there is no overlap. For detection in mass-cytometry the cells are vaporized and the mass of the reagents bound to the cell is quantified by mass spectrometry.

However, neither flow nor mass cytometry can perform dynamic analysis of single cells and most instruments do not allow observation of spatial localization of fluorescence within a cell. These limitations are addressed by another common technique for dynamic single-cell studies – optical microscopy. By imaging one cell at a time optical microscopy enables monitoring of processes such as migration, proliferation, and cell-cell interactions. It also allows for staining of cells to correlate for example functional properties to expression of cell-surface markers. However, tracking multiple single cells manually over time is difficult since cells easily disappear from the field of view unless imaging is performed with low resolution.

A recent development is the combination of optical microscopy imaging and fluorescent flow cytometry (e.g. Imagestream). The addition of two-dimensional images provide new data, which has proven useful to monitor for example morphology of cells or spatial localization of proteins within cells. This method provides both statistical and throughput advantages compared to conventional optical microscopy-based methods, but is still limited to only a snapshot in time.

Other techniques commonly used for single cell analysis include: laser scanning cytometry where individual cells are imaged and quantified in the tissue; capillary electrophoresis for efficient separation and sensitive detection of whole cell or subcellular samples; and laser capture micro-dissection for excising and separating single cells from tissue for further analysis. Unfortunately, the major drawback of almost all of the techniques mentioned above is the low throughput.

1.2.3.2 Minaturized devices

With the aim to address the low throughput a plethora of miniaturized devices for single cells studies have been described. They all try to solve the challenge of adequate parallelization to enable statistically meaningful conclusions. Most of them are based on cell separation using different techniques; some trap cells using flow systems, others use suction immobilization, or are based on sedimentation of cells into separate wells. Many of the techniques for
analyzing large numbers of cells in wells, have successfully been applied to several adherent cell types \(^{75, 79}\), but have proven more challenging for long-term imaging of motile suspension cells. Various capturing techniques have been applied to solve this problem; functionalization of shallow wells' interiors with specific ligands or antibodies \(^{74, 85}\), physical confinement via lids \(^{86}\) or tight well dimensions \(^{80, 87}\).

A specialized technique for physical confinement with lids is called microengraving, it was originally developed for screening of antibody producing single cells to accelerate hybridoma technology, and is based on soft-lithography and PDMS-based microwell chip. This technique has been successfully applied to studying for example primary T cells from HIV-patients and NK cell heterogeneity \(^{88, 89}\). It offers the advantage of being able to evaluate the cytokine secretion profile of the immune cells being studied, but due to environment the physical confinement the experimental time is limited to a few hours.

An alternative method to trap live cells without the requirement of microwells is droplet microfluidics, the technique itself was described long ago\(^90\), but has later on been applied for entrapment of live cells. Here live cells are encapsulated in microdroplets of medium suspended in an inert oil which allowing the passage of gases to the cells\(^91, 92\). These droplets can then be passed through an optical path for automatic detection within the microfluidic system. A few reports describe how these droplets can be useful for fairly long-term live cell assays, up to 11 hours\(^{93, 94}\). An advantage of this system is that the cells of interest are readily accessible for further analysis, for example PCR.

However, none of these techniques support real long-term studies including cell proliferation (except for droplets) and also offer limited possibility to study, e.g. migrational behavior and multiple cell-cell interactions of untouched cells. These are all important factors to consider when monitoring the immune system, which is highly diverse and poly-functional.
2 Materials and Methods

The material and methods of relevance in this thesis are also described thoroughly in each paper. Since this thesis is focused towards the newly developed method, I here discuss some parts in more detail that did not fit the requirements for the scientific journals. The actual microwell techniques that were developed in the course of this thesis work will be presented in the results section.

2.1 Microwell Chips

To address the problem of long-term imaging of living cells a series of differently sized multiwell microchips were designed and fabricated with the methods described above. A silicon mesh was etched by DRIE and subsequently bonded to a glass slide, creating an array of open silicon microwells where the bottoms of the wells can be imaged by an inverted microscope. Also an inverted version was etched in silicon, functioning as a mold when casting soft silicon rubber chips.

For single-cell screening a large number of interactions are needed in order to obtain reliable statistics and not just random events. Therefore some chips were designed to contain as many wells as possible, resulting in a dense pattern of wells with narrow walls in between. This strategy also aimed towards minimizing the number of cells outside any well. For applications where the migrational behavior of the cells and multiple interactions with different cells are of interest, larger wells were designed.

All type of chips were first primed by adding medium to the wells. Cells are then seeded onto the chip and left to sediment randomly, for the larger wells more controlled seeding with a pipette is also feasible. Cells can then be grown in the chips for up to a week when placed in an incubator, or be used immediately upon seeding.

To optimize the geometries of the wells for different applications, computer simulations of cells interacting in wells were performed (Paper I). By measuring the expected time to cell-cell interaction in different well sizes and assay set ups, general ideas of suitable designs to fabricate were obtained. These were then evaluated and compared to the simulations.

2.1.1 PDMS chips

Polydimethylsiloxane (PDMS) is an optically transparent soft elastomer widely used in microfluidics and other miniaturized lab-on-a-chip technologies. Low auto-fluorescence and biocompatibility makes it a suitable material for many biological assays including imaging. Its softness is an advantage because it enables easy manipulation of cells inside the wells; the rationale being that the possibility to pick out cells of interest for further cultivation or experiments is
2. Materials and Methods

highly desirable. Another benefit is the low production cost compared to etching in silicon. Fabrication of a mold to cast the PDMS is always necessary, but as this can be reused many times the cost of a single chip is reduced. Single use chips are then economically feasible to fabricate, hence reducing the workload on the practitioner.

However, the casting process limits the obtainable geometry of the wells as the cured PDMS has to be peeled of the mold without breaking. Thin and long structures are brittle; to overcome this we increased the thickness of the walls and limited the depth of the wells to 100 µm. Another drawback with the PDMS microwells is its optical properties, as imaging through the bottom of the wells cannot be done with high-resolution microscopy. This can be solved by sealing off the wells with a cover glass and invert the whole sandwich to image through the new glass well-bottom.

Unfortunately untreated PDMS is highly hydrophobic, causing medium to be expelled from the shallow wells. Therefore pretreatment of the PDMS is necessary, two different approaches were made; plasma treatment and fibronectin coating.

2.1.2 Silicon chips

Silicon chips of different geometries were fabricated with the microfabrication techniques described before. In order to fit in a common holder the outer dimensions of all chips were the same, 22×22 mm², a common size for microscope glass coverslips. The structures were etched in a standard silicon wafer which is 300 µm thick and bonded to a 170 µm glass, giving a total thickness of 470 µm. Following etching some of the structures were oxidized at 1000°C for 24 min to achieve a 200 nm thick SiO₂ layer. Oxidation had to be carried out before bonding to the glass as glass melts at the oxidation temperature.

<table>
<thead>
<tr>
<th>Design</th>
<th>Well width, w (µm)</th>
<th>Wall thickness, x (µm)</th>
<th>No of wells</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>20</td>
<td>90 000 - 102 400</td>
<td>Screening</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>30</td>
<td>32 400 - 40 000</td>
<td>Screening</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>100</td>
<td>100 - 600</td>
<td>Ultrasound manip.</td>
</tr>
<tr>
<td>4</td>
<td>450</td>
<td>350</td>
<td>400</td>
<td>Migration</td>
</tr>
<tr>
<td>5</td>
<td>700</td>
<td>100</td>
<td>400</td>
<td>Migration</td>
</tr>
</tbody>
</table>

Table 1. Silicon chip designs and applications. Five different designs of microwell chips with different dimensions and geometrical properties have been fabricated and tested for various applications.

We end up with a silicon grid, in the form of an array of 300 µm deep microwells covering the chip. The well depth is important as this is what isolate the individual wells and prevent cells from moving in between wells. The number of wells and the bottom imaging area differs depending on application, but the depth always remains the same. Well size, distribution and wall thickness were
optimized for the specific experimental applications in mind. Up till now 5 different designs have been fabricated with varying well sizes (30-700 µm) and wall thickness (20-350 µm) yielding chips with the number of wells ranging from 100 to 102 400 total wells (Table 1).

For the screening wells all individual wells were indexed with letters and numbers, indicating row and column respectively. Indexing greatly facilitated finding and returning to wells of interest when using a high magnification objective for imaging, as well as making screening of subparts of the chip possible. The arrays of wells on the chip are also subdivided to optimize for screening without losing information. During screening a large number of individual images are needed to cover the whole or larger parts of, the chip. These can either be stitched together or kept as individual images, but should preferably not be cut in the middle of a well. For example, the 50 µm screening chips are divided into 20×20 smaller arrays, each containing 9×9 wells, designed to be imaged with a 10× objective.

Evaluation of some of the designs has been performed, where the main part of this thesis is focused on the use of screening chips and a smaller part on migration chips. Chips for ultrasonic manipulation of cells are not in the scope of this thesis but have been evaluated elsewhere 96, 97.

2.1.3 Holder
A custom made holder was designed and fabricated in order to facilitate handling of the chips (Fig 3). First a titanium bottom holder was hollowed with a 0.2 mm thin encircling flange supporting the chip. The flange had to be very thin and flat in order not to obstruct the movability when using a high-magnification oil-objective. Titanium was chosen because of its heat conducting properties and its well-known biocompatibility. The first generation holders were made out of aluminum, but were interchanged for titanium because of the potential leakage of harmful metal ions into the medium.

The top lid was made in the transparent material poly-methyl methacrylate (PMMA) and has a 20 mm x 20 mm hole in the center. This open system allows addition of material, e.g. cell culture medium, fluorescent probes or drugs, to the cells during imaging. Four magnets are placed in the bottom holder and another corresponding four in the top lid, making it possible to easily clamp the device together tightly after assembly. To prevent leakage between the chip and the lid a PDMS gasket is used, together they form an inside cavity of the holder. This way a substantial amount of medium, 1-3 mL, can be added on top of the chip and thus permit long-term culturing of cells without evaporation or loss of nutrients.

The dimensions of the holder has been made so that the bottom part fits closely in an environmental chamber, and a 35 mm Petri dish lid can be used to cover the open top when keeping it in unsterile environments.
2. Materials and Methods

2.1 The custom made holder facilitates long-term imaging and cultivation of cells inside the chip. The chip is sealed off for sterility but still easily accessible for medium exchange or addition of reagents. Four magnets clamp the holder together with the PDMS gasket tightly sealing the chip to prevent leakage of medium. Figure adapted from Paper V.

2.2 Automatic data analysis

The large amount of data generated while screening a full chip is too large to be efficiently analyzed manually - more than a hundred thousand events may take place in the smaller screening chips! In our first evaluation of the chip (Paper I) only smaller subparts of the chip were analyzed manually for proof of concept. In our second study (Paper II) the first generation of automatic analysis was implemented and evaluated. However, during further development of the automatic counting a new approach was implemented. This method was found to be more user-friendly (and accurate), and is used in the second generation program presented in Paper V.

The main challenge to overcome for an algorithm is how to classify what a single cell is with accuracy. The human eye and brain usually have no problem distinguishing one large cell from a cluster of two smaller cells, but a program needs to be supplied with different criteria in order to classify objects. Both programs struggle with two main tasks: 1) the first step is to separate the objects from the background noise, 2) and then to adequately classify the objects to count the number of cells. The different strategies behind the two programs will briefly be covered here, more in depth information can be found in Paper II and Paper V respectively.

2.2.1 First generation program

In the first generation program Otsu’s method was used to separate the objects from the background; a fast method to separate two classes of pixels (here foreground and background), based on finding the optimum threshold that minimizes the weighted within-class variance \(^98\). Clustered objects will not be
2. Materials and Methods

separated by this method, but since objects have been separated from the background, it is now possible to extract some cellular features. Area, perimeter and compactness were selected as morphometric features to make up the training database. These were calculated based on the pixel values and their spatial arrangement within the object. Intensity features were not considered as some objects were unfocused and there was significant variation in object intensity.

An additional step of approximation was then needed to distinguish between the two classes of data (single cells and clusters). Since the number of single cells is greater than the number of clusters, we assign single cells as our database. This database is based on a large data set of manually analyzed single cell objects, which has a Gaussian distribution and a mean value. For each of our sample test points we then check the estimated Mahalanobis distance \(^99\) to the mean of the training data set of single cells. Together with a predefined threshold this can be used to discriminate between the two classes; single cells and clusters. If the distance is greater than the threshold this test point will be regarded outside the database class (single cells class), so it will be classified as a cluster object. If an object was classified as a cluster, we approximated the number of single cells by dividing its area with the mean value of the area for all single cells. Finally, the number of all cells in each well was counted.

2.2.2 Second generation program

While the first program relied on training databases with multiple cellular features, the second generation program instead used a built-in MatLab algorithm that uses only circularity as a mean to distinguish cells.

First this software identifies the actual wells by a combination of the trans-illuminated DIC-channel and the inverse of the red channel - in which there is a lot of autofluorescence from the microchip itself. When the pixels containing wells are identified, the program searches through all of the fluorescent channels one by one for cells. First it removes all objects outside wells or with too low fluorescent intensity, and then the remaining objects are convolved with a Gaussian filter to improve circularity and simplify the separation of multiple-cell objects. The Gaussian is chosen to be approximately the same size as the cells; hence different filters have to be used for NK and target cells because the NK cells are smaller by nature.

The MatLab program then applies an algorithm based on the circular Hough transform to search for roughly circular objects\(^100-102\). The size range of objects searched for is varied between the size of target cells and NK cells. The software then returns a numbered map of the found wells, the found objects, and the number of objects in each well. In addition, the program also returns a figure of goodness describing the chances that the software had made a correct determination of the number of cells for each well. This figure is based on complicating factors such as large numbers of cells in a single well; the
existence of large contiguous objects (likely to be multiple cells), bright objects
that could not be identified as cells and amount of overlap of identified cells.

2.3 Cell culture

Both cell lines and primary human cells have been used in our studies. The main
target cell lines used were adherent human embryonic kidney (HEK) 293T, K562
and 721.221 suspension cells (referred to as 221). Both K562 and 221 target
cell lines lack expression of MHC class I and are therefore susceptible to NK cell
killing, whereas 293T express low levels of HLA-E and other types of MHC I. All
cell lines readily proliferate in cell culture.

2.3.1 Cell lines

The 221 cell line is an Epstein-Barr virus (EBV) transfected B cell line. EBV is a
human herpesvirus primarily infecting B cells causing infectious mono-nucleosis,
also known as kissing disease. The infection immortalizes the cells and results in
an autonomously proliferating lymphoblastoid cell line that carry latent viral
genomes\textsuperscript{103}. This feature has been exploited by scientists and nowadays also
other cell types than B cells can be transformed in a controlled fashion\textsuperscript{104}. Some
221 cells were also transfected to express one type of cell surface MHC class I,
either single HLA-Cw6 or HLA-Cw6 coupled to GFP\textsuperscript{105}. This renders them
unsusceptible to NK cells expressing the corresponding KIR, which for HLA-
Cw6 is the inhibitory receptor KIR2DL1. Since MHC class I is involved in the
immune synapse formation GFP-labeling makes it possible to follow the
clustering of molecules at the cell-cell interface.

The other target cell line used was the human embryonic kidney 293T
(HEK293T) cells. HEK293 cells are easy to grow and transfect readily, and are
therefore widely used in cell biology research. They were generated from
cultures of normal embryonic kidney cells by transformation with sheared
adenovirus 5 DNA in the early 70s\textsuperscript{106}. In addition, the 293T cells contain the
Simian Vacuolating Virus 40 (SV40) large T-antigen, a protein which allows for
amplification of any transfected plasmids containing the SV40 origin of
replication. For a long time it was generally believed that the 293T cells were
generated from either fibroblasts, endothelial or epithelial cells, all of which are
abundant in kidney. It has now been shown that these cells share many
properties with immature neurons, suggesting that the adenovirus instead
transformed a neuronal lineage\textsuperscript{107}. As these cells were only used as cancer target
cells or as a model for cell growth their disputed origin does not affect our
experiments.

We also used the K562 target cell line – a human erythroleukemic cell
line, and an often-used target for studying NK cell activity. It was originally
established in 1975 from the pleural effusion of a patient with chronic myeloid
leukemia (CML) in terminal blast crisis\textsuperscript{108} and was later described to be of
erythroid origin\textsuperscript{109}. K562 are MHC I deficient and sensitive to killing by NK cells.
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As a human NK cell model we used the YTS subline of the YT leukemia cell line\textsuperscript{110}, which normally do not express any known KIR. Both wildtype and cells transfected with the inhibitory receptor KIR2DL1 were used for proliferation studies and for synapse formation respectively.

All of the cell lines were maintained in RPMI-1640 medium supplemented with fetal bovine serum, L-glutamine, and a mixture of penicillin and streptomycin. The cells were cultivated in an incubator providing optimal growing conditions, 37°C with 5% CO\textsubscript{2}, and split approximately every 3 days or when needed.

2.3.2 Isolation of primary human NK cells

In order to study cellular heterogeneity we also used primary NK cells. These cells are polyclonal since they are isolated from human peripheral blood, and hence represent the diversity of a “normal” NK cell population. We also found that the wildtype YTS cells displayed poor cytotoxicity and therefore the much more potent primary cells were used in all of the killing assays. Cell lines are a much more controlled model system where cells in a population are assumed to be more or less similar.

Primary human NK cells were acquired either from lymphocyte-enriched buffy coats or fresh blood from healthy donors. First all peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on a Ficoll-Paque Plus gradient. The serum fraction was also gathered and heat inactivated for later cultivation. To isolate NK cells from a mixture of PBMCs negative selection via magnetic bead sorting was used. An antibody cocktail recognizing all PBMCs except NK cells, where the antibodies are coupled to small magnetic beads, is first added to the cells. When passing the cell suspension through a column placed in a strong magnetic field all unwanted cells will be trapped and only the NK cells eluted. Negative selection is preferred to positive selection - where the antibodies recognize the cells of interest - because the cells are less tampered with and antibody binding may activate or otherwise affect the cells in an uncontrolled way. Normally positive selection yields higher purity of cells than negative selection.

After isolation the primary cells were cultivated at 37 °C and 5% CO\textsubscript{2} in Iscove’s modified Dulbecco’s media (IMDM) or RPMI-1640 supplemented with human serum, L-glutamine, non-essential amino acids, sodium pyruvate, antibiotics and the cytokine human Interleukin-2 (IL-2). IL-2 is added to induce cell expansion and has also been shown to greatly increase purity and cytotoxicity\textsuperscript{111}. Primary NK cells are dependent on cytokines when cultured for more than a few days, or they will die.
3. Results

The overall aim of this work was to go from evaluation and development of an untested technique to implementation of a screening system for investigation of immune cells. First the general technique of cell culturing and imaging in the microwell system, labeling strategies, and microscope issues had to be worked out. Then we could start testing the microwell platform with a couple of different approaches for live single-cell assays. My work has been focused towards the screening wells - which have been used both for time-lapse imaging and the pure screening approach with only a few imaged timepoints. Once the method was established the main focus has been to make the screening approach work efficiently for investigating serial killing NK cells. Importantly, the method itself has been tweaked over the course of the study as new ideas have been implemented and initial problems have been solved. Here I will go through the most important steps during development as well as the biological results we have obtained.

3.1 Cell culturing in microwells

The very first study of our microwell platform was to test its biocompatibility. A test to see if cells proliferated normally was performed by culturing a few types of cells both in microwells and in a conventional petri dish. All cells were counted at three timepoints (0h, 24h, and 48h) and by comparing the proliferation rates we saw not only if they proliferated normally but also whether they were confined in the wells or not (Paper I) and Figure 4.

PDMS wells were first tested and turned out to be too shallow to trap the cells for a long-time. During long-term imaging approximately 10-20% of the cells migrated between the wells. To eliminate this problem a confinement technique was applied (Paper I), were a second glass slide was used to cover the wells, hence confining the cells. This scheme worked well for confinement, but ultimately limited the accessible volume of medium and thereby possible imaging time. The technique chosen to make the surface more hydrophilic turned out to have a large impact on cell survival time, where treating the PDMS with fibronectin resulted in increased survival time compared oxygen plasma treatment. Although imaging is still possible for shorter periods of time (<6 hours) the PDMS chips were abandoned because they proved to be less robust and have a more limited use compared to the silicon chips.

The silicon chips are much deeper than the PDMS, 300 µm compared to ~100 µm, and this increased depth makes almost all cells stay confined. However, depth in combination with narrow well geometry results in diffusion being the only way nutrients and gases can be exchanged at the bottom of the wells. Hence, we were concerned that this would be a limiting factor that may affect cell growth, especially after a few days of proliferation. Three different cell
lines were simultaneously cultivated in separate microwells or petri dishes and the proliferation rate calculated. It turned out to be similar even after a few days proliferation. Cells seem to thrive even after a week of cultivation though they were no longer possible to count (data not shown).

![Figure 4. Normal proliferation rate of both adherent and suspension cells in silicon chips. Calcein stained 221 (suspension) and 293T (adherent) cells were cultivated in 50 μm screening wells for 3 days and imaged every 24 hours. Images show the same set of wells for all the 3 timepoints.](image)

During the course of testing the microchips one important finding was made; the smaller wells required oxidation during fabrication - this extra step turned out to not be required for the larger wells. Unless oxidized the cells started to swell within hours and died, the reason for this is still unknown. Oxidation does have an additional advantage as the chips become inert to some chemical treatments; like concentrated NaOH. Cleaning the chips with concentrated NaOH may occasionally be necessary if the chips are very dirty and some cell debris proves impossible to remove with the normally used milder sonication cleaning. This since the smaller wells in general require more thorough cleaning, owing to both the greater total number of cells used and its geometry, the additional oxidation step turned out to be beneficial in more than one aspect.
3. Results

3.1.1 Influence of the chip’s geometrical properties

The chip’s geometrical properties have a large influence on what type of assays it is suitable for. As expected, we found that the smaller screening wells were indeed suitable for fast cell-cell interactions, e.g. synapse formation, and cytotoxicity studies. In wells with both NK and target cell, most cells had formed synapses within an hour after seeding, as was expected from a series of simulations of the cells expected time to cell-cell encounter in differently sized wells (Paper I). Simulating the cells behavior gave a good estimate of appropriate well sizes to fabricate in order to make screening more efficient.

The square geometry is a scheme to maximize the well/wall area ratio of the chip, hence minimizing the number of cells being trapped on top of the chip during seeding, in addition to make the wells compatible with the microscope format. Other proposed geometries are e.g. circular or hexagonal wells in a beeswax structure. Circular wells waste a lot of wall area if straightly aligned in rows and columns, and the tighter positioning of the interwoven beeswax structure does not fit the square output images generated by the microscope. Straightly aligned square wells not only maximize the amount of image data per stored pixel, it also facilitated indexing.

The size and distribution of the wells were also optimized for imaging with certain magnification objectives. For imaging of large migration wells a 20× objective was used, covering only one well at a time, while the screening chips are divided into 20×20 smaller arrays designed to be imaged with a 10× objective. Any series of images covering a larger section of the chip can in the end either be stitched together as a tile or kept as individual images, where keeping the individual images is less time consuming during image acquisition. However, cutting in the middle of a well should always be avoided to prevent loss of information – only whole wells can be properly analyzed and perfect alignment when stitching the cut images together is hard to obtain.

3.1.1.1 Confinement during long-term cultivation

As mentioned in the technology section we abandoned the PDMS chips because of their lack of easily accessible confinement. The need to cover the chips and clamp the sandwich together not only limited possible imaging time but also introduced a cumbersome extra step into the assay. We therefore concluded that a well depth of 100 µm is not deep enough to keep the cells properly confined. Other strategies to keep cells confined in shallow wells have been proposed by others before, like antibody trapping or suction, but none of them were suitable for our assays where we wanted the cells to migrate freely and be “untouched”.

For the much deeper, 300 µm, silicon microwells lack of confinement was a minor issue. The initial problem of cells being stuck on-top-of the chip or halfway down the wells, only to later fall into the wells when migrating, was more or less resolved by introducing a washing step after seeding. This was possible
without disturbing the seeded cells because the medium inside the small wells of the screening chips was not affected due to their narrow geometry. Also, with the second generation chips' smoother walls cells no longer got stuck half-way down the wells.

With the current method, a very small number of cells still migrated between wells but the vast number of cells stayed in the well they were seeded into. Lack of confinement is naturally more apparent when working with suspension cells and very motile cells, for adherent and less motile cells it has not posed any problem. Confinement data is only available for the smaller wells as it has not been thoroughly evaluated for the large migration wells. During the migration studies we both have fewer cells and all the individual cells are continuously tracked, preventing them from changing well without being noticed. Also we never experienced lack of confinement to be an issue in the migration wells and it has therefore not been addressed.

3.1.2 Imaging properties and labeling limitations

One of the first limitations we encountered when evaluating the method were issues concerning the fluorescent labeling. For real long-term imaging stable fluorescent dyes are needed in order to keep track of the cell during the whole imaging session. Especially for the automatic tracking homogenous and stable dyes are desired. Mainly because the program uses features derived from the fluorescent channels to identify cells, and as the fluorescent image of homogenously stained cells has more cell-like features, e.g. roundness and appropriate size, they are not erroneously perceived as noise or cell clusters.

Many of the used dyes show great performance for a few hours, but over time they either bleach or slowly leak out of the cell. This is especially true for the viability dyes; calcein AM and calcein red-orange; since they are freely dispersed in the cytosol and do not form any chemical bonds to any part of the cell. The membrane staining dyes are more stable but also less homogenous and can sometimes affect the cell in unwanted ways. For example, we found that the migratory behavior, killing potential or both was affected for the primary NK cells, and therefore these types of dyes are only used for target cells. The amount of dye a cell retains is also dependent on cell type; some cell types retain the dye better than others. Primary NK cells for example seem to lose their fluorescence faster than any of the tumor cell lines. For most of our purposes, screening and migration assays are ≤12 hours, and the retained fluorescent intensity is usually sufficient to adequately analyze the cells. This however needs to be considered when considering real-long term assay (days), an example of this intensity loss is visualized for calcein AM stained cells cultivated for 48 hours (Fig. 4).

For successful imaging we also need to consider the limitations of the microscope and the computational tools used for the later analysis. The microscope used ultimately sets the limits for the type of imaging that is possible. Usually, imaging is a tradeoff between resolutions in a number of
dimensions depending on the microscope’s performance: for example time-, area- or pixel-resolution. A faster microscope allows either scanning a larger part of the chip within a set time, or take images more often of a set area. Also we cannot generate infinite large files because they become unmanageable; instead the generated data must be of good enough resolution for what we are interested in analyzing. It is a balance and we need to consider the limitation of the set up and how to use it in the best possible way before we start our experiment.

3.1.2.1 Time-lapse imaging

Time-lapse imaging in our microchip platform turned out to work very well, also for long imaging sessions. Cells were viable for the whole imaging period (up to 12 hours) and seemed to thrive as they both proliferated and remained a healthy morphology. The chip was submerged in medium in the holder, which indeed kept it from drying out in the heated environmental chamber. Also, we did not experience any major problems either with focus or gravitational drift during the long-term imaging, meaning that we have a stable platform for continuous imaging assays.

However, for long-term imaging the stability of the fluorescent dyes have to be considered and compensated for (and possibly eventually exchanged if a new dye with more favorable properties will become available). For example, for imaging sessions run over night the microscope settings had to be tweaked so that the specimen is overexposed in the beginning of the image session in order to still be visible at the end of the movie. Generally we need to compensate slightly more for time-lapse imaging, compared to screening, as the continuous imaging add bleaching of the dye to the general leaking. An alternative way would be to program the microscope software to increase the laser power over time, but with our current set-up that is not possible.

With all of these requirements fulfilled obtaining the type of time-lapse movies we desired was feasible. We did both long-term cytotoxicity movies in small wells with primary NK cells and 293T tumor targets (Paper I) and migration movies in large wells with success (Paper III-IV) and \(^\text{172}\), and with K562 targets in Paper V.

3.1.2.2 High resolution imaging

We tested the microwell conditions for high-resolution imaging by looking at synapse formation between 221-Cw6-GFP cells and the NK cell line YTS-Kir1, these cells form an inhibitory IS that results in Cw6-GFP clustering at the cell-cell interface. One main advantage of doing high-resolution imaging of living cells in small microwells is that they are trapped in one spot, and thereby prevented from leaving the field-of-view during scanning. This is due to less drift in the medium itself, which can be caused by thermal motion or gravitational forces on the chip, but also because other interacting or passing-by cells are hindered from moving the imaged cell. This is especially apparent when doing
3. Results

High-resolution confocal imaging because performing a z-scan through the specimen takes time and for a well-turned-out 3D-reconstruction the specimen movement during scanning should be minimized. Often this type of imaging is therefore performed on fixed specimens to achieve 3D high-resolution, unfortunately fixation rules out imaging of dynamic events. Hence, a very fast microscope is a prerequisite to perform live cell confocal imaging of fast dynamic events.

High-resolution confocal imaging of immune synapse formation between the 221-Cw6-GFP and YTS-Kir1 cells was successfully performed, and 3D-reconstructions subsequently made. A major advantage of the numbered array-like structure is how one can first have an overlook with a less magnifying objective to identify wells of interest and then go close on those wells with a high magnification objective. This way it is also always possible to go back to the same well at a later time-point, which enables the possibility of performing high-resolution time-lapse imaging of several wells in parallel.

3.1.2.3 Screening

In this approach the idea is to use the small 50 μm screening well chip to confine the cells at a set location and only image the chip at two or a few time-points (Fig. 5). Because we only image at a few timepoints it is possible to scan the whole chip, acquiring images from all 400 locations, and subsequently analyze them. For most of our applications a single effector cell together with a few target cells are desired in each well. This way we know that a particular NK cell is responsible for the outcome of that well. Because seeding of cells into these tiny wells leads to random distribution, approximately 20-30% (max) of the wells will end up with the desired distribution. However, since we have such large total number of wells, 32 400, there will be a sufficient number of eligible wells although not all of them have the desired distribution. Some of the other wells may be usefull as internal control for spontaneous target cell death (wells with no effector cell, only targets). In order to effectively analyze the vast amount of data generated by a full screen chip an automatic counting program will be required. However, for all proof-of-concept studies and to enable evaluation of the accuracy of the automatic counting manual analysis have been used.

The very first tests of screening together with automatic counting worked out well (Paper II). The holder enabled automatic imaging of the whole chip little focus-loss and kept the cells submerged during the full imaging session. Subsequent processing and automatic analysis of the acquired data was also successful. In Paper II we took the first steps towards screening when we evaluated clonal expansion of GFP-labeled 221 cells grown under antibiotic selection at two different time-points. However, there was no cytotoxicity screen where data from two (or more) time-points were compared to evaluate effector cell activity. This has been the focus of my recent work, and has resulted in Paper III-V and a manuscript in preparation. The recent experiments needed to
fully evaluate the system and use it to retrieve biological data are partly presented in Paper V and discussed in this thesis.

**Figure 5. The cytotoxicity screening process.** A) Schematic over-view of the different steps in the screening process. The In-chip steps can be iterated as many times as wanted. B) Example of screening images showing the same part of the chip at two different timepoints, 0 and 6 h. NK cells labeled with calcein red-orange (blue) are mixed with K562 target cells labeled with calcein AM and DDAO (green or red), when the targets are killed they go from green to red. One well is chosen to show how the automatic analysis is performed in principle, here only for the blue and green fluorescent channels. MatLab identifies and counts the objects, schematically shown as red dots, based on an inbuilt algorithm. A is adapted from Paper V.

In the screening assay the small wells are imaged two to three times; first directly post-seeding of cells to register each well’s starting cell content, and second after some time has passed (approximately 6-12 hours) to allow for cell-cell interactions (Fig. 5). We observed a gradual loss of fluorescent intensity over time. Since the chip is only imaged a few times bleaching of the dye is not likely, instead the insufficient retaining of the viability dye is the main limitation when it comes to detection and assay time. Because the imaging sessions take place at different time-points, the microscope settings can be optimized accordingly - as long as the dyes are still visible. For up to 24 hours compensating the loss with slightly increased excitation is sufficient to achieve good quality images, but for longer incubation times one may have to consider other strategies for long-term labeling. This is especially true for the small primary cells; NK or T cells, which loose their fluorescence much faster than the cell lines. However, in our application target cell death is the main read-out when studying the cytotoxic potential of NK cells or performing a T cell screen, and for this purpose our labeling strategy turned out to work very well.
For the NK cytotoxic screening we eventually decided to analyze 3 time-points, 0, 6, and 12 hours (Paper V and manuscript in preparation). Initially this was done in order to determine at what time-point you could expect to detect cytotoxic behavior from the majority of active effector cells. In Paper V it became apparent that a substantial part of the killing between 6-12 h was actually carried out by 'new' killers, roughly half for K562 and one third for 293T (Fig. 7B), which was more than expected. This emphasizes the importance of having long-term experiments in order to catch the whole range of active effectors. For the later screening experiments aimed towards identifying serial killing NK cells we kept the 3 time-points to detect as many serial killers as possible. The importance of the last time-point in regards to this is not fully clear yet, as the data is still in need of thorough evaluation.

What did become clear in Paper V is the importance of the first time-point $T_0$ in connection to accurate analysis. Two main features will impose on the result since the screening approach only gives information from snap-shots of time. First are the dead-when-seeded target cells, which are impossible to distinguish from the targets killed before completion of the first $T_0$ scan. In general, a large fraction of the active NK cells start killing off their targets immediately after seeding, and scanning the whole chip creates a lag-time between initiation of experiment and the capturing of image $T_0$. This becomes apparent especially at image frames with higher numbers (hence longer lag-times), where the fraction of dead target cells at $T_0$ is much larger in wells containing NK cells than in ones with only target cells (Paper V, Fig S1B). Second is the spontaneous death of target cells that occurs during the experiment, this however seems to be a fairly small problem since it only effects a few percent of the cells in the evaluated experiments (Paper V, Fig S1A).

To solve the second problem, all experiments with too high spontaneous target cell death were excluded - therefore we believe it is reasonable to ascribe all target cell death that occurs between 0-6 hours to the effector cell in that particular well. To diminish the problem of dead-seeding it is vital that the target cells used are in good condition before staining. The percentage of dead-seeded cells also seem dependent on the target cell line used; in our experiments generally a higher percentage of dead-seeded target cells were present for K562 compared to 293T targets, and even higher (up to 20%) for the C1R/A11 target cells used in the T cell screen (Paper V). Spontaneous target cell death represents an important issue that may limit the sensitivity of the assay.
Technical Challenges
Screening of the whole chip also resulted in new technological challenges more related to the hardware used. Nonetheless they needed to be addressed during the development of the method. Because we in total image such a large area (microscopically that is), up to $15 \times 15 \text{ mm}^2$, the chip need to be perfectly flat or the wells will gradually go out-of-focus when moving along the chip. We have continuously upgraded the type of chip-holders used and for the newer generation of holders this problem has diminished. Imaging of a large area also takes time, scanning the full chip require 400 individual images when using a 10× objective, approximately 1 hour in our current set up depending on the required quality of the images. All of the images are taken post-seeding, and in the last scanned wells some action may already have occurred when the first image is taken. One strategy to minimize this problem is to keep the cells cold until just before seeding, thereby giving them a slow start – though this approach has not been thoroughly evaluated.

Another challenge with the screening approach has been related to the limitations of the microscope. Two different systems have been used, Olympus for the T cell-screening project and Zeiss (LSM Meta) with the ZEN operation program for the NK project. The Olympus microscope stage had the advantage of being almost completely flat so that approximately 90 % of the wells were in focus, and also to have a very versatile program allowing for easy set-up for a full-chip screen. The Zeiss set up the ZEN-program was not as straightforward because of limitations of the software itself and problems to get all wells in focus. Therefore information from many wells was lost using this set-up. Both set-ups lack an autofocus that is functional within a time-series of multiple positions – which would have solved the focus issue. Despite this we still retrieved more-than-enough information from both set-ups – more than could be handled manually and some still in need of automatic analysis.

3.2 Single cell analysis
Both for cytotoxicity and migration studies there is a need to analyze single cells. In the cytotoxicity screen setting an accurate number of effectors, dead and living target cells need to be determined for each and every well at all timepoints. This is done much more efficiently with an automatic counting program, although manual counting still has a lot of benefits when it comes to accurately determine cell numbers in ‘difficult’ wells. During the course of this thesis two different versions of programs have been made. The first we used in Paper II, and the second in Paper V.

For the non-screening approach, cytotoxicity and migrational behavior is continuously followed by time-lapse imaging where the individual effector cells were instead tracked either manually or with Volocity software (Paper I and IV). Some objects that were particularly difficult to track needed both.
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3.2.1 Detection of live/dead cells

In all studies we used a labeling strategy based on fluorescent dyes to detect live/dead target cells. Sometimes this was used in combination with visual morphological changes interpreted as signs of target cell death, such as apoptotic blebbing or membrane ‘explosion’ (Paper IV). Fast membrane bursting leads to almost instant loss of the fluorescent cytosolic dye (calcein) used for identifying living cells, whereas apoptotic death leads to slower loss of calcein. In contrast, the only dye that is retained is the Far-red DDAO bound to primary amines present everywhere in the cell. In most experiments DDAO showed an additional beneficial feature by not just being retained but actually increase in brightness when the target cell died. It was also noticed that the target cells that were dead-seeded were already many times more bright red than the cells that were initially alive, most likely due to them accumulating much more dye during the labeling process. In the future this feature may very well be a promising way to distinguish the target cells that were dead-seeded from those that were killed during the experiment.

The NK cells were only labeled with a cytosolic dye because there is still no permanent dye we have tested so far that does not inflict on their migratory behavior available. Therefore it has been challenging to accurately determine the fate of the NK cell itself in the screening setting. When they are alive and visible their number is easily scored, but when they are not present at some timepoint it is unknown whether this is because they are dead or if they momentarily migrated out of focus – which some of them do for shorter periods of time. With the 3-timepoint strategy this is more easily evaluated since a majority of cells return if they are not dead. In experiments with very good staining of the NK cells, it may also be feasible to perform analysis of the fate of the NK cells. However, this type of dead-NK analysis is something that the automatic counting program likely will not carry out very well as it only bases its counting on the fluorescent channels and not the transmission image.

The automatic counting program counts the cells in all fluorescent channels, where all target cells should be red, but only the living ones should also be green. Then the program will rate the cells depending on their green/red intensities, dead cells being mostly red and alive cells being mostly green. This is the same strategy used for manual counting, but usually manual counting also take the morphological features into account for the difficult cells. Though the second generation program has not been extensively tested it is a significant improvement that the new program also returns a figure of goodness, describing the chances that the software had made a correct determination of the number of cells in that particular well. When we perform a full-chip screen where manual analysis is impossible we can now chose to exclude all wells with a figure of goodness below a certain threshold. In this case we will be able to analyze data much more efficiently and still be able to trust it.
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3.2.2 Tracking of individual cells

The initial cytotoxicity studies involved a combination of manual and automatic tracking of individual cells to increase accuracy. Manual tracking is very time consuming but still the most accurate alternative as a variety of events misleading the automatic tracking can occur. For example it proved important not to have too high density of cells to prevent cells from occluding other cells. In the first study (Paper I) the migration pattern of the cells was not registered but only the length, outcome and number of interactions each effector cell had with all encountered target cells. The stationary 293T cells were used as targets in all cytotoxicity studies; their fluorescence was recorded over time as a measure of viability using the Volocity software. However, they are not completely stationary which had facilitated the analysis, but rather crawling slowly. Sometime this behavior, or that target cells were moved around by the motile effector cells during interaction, caused problems with cells that escaped tracking.

3.2.3 Automatic counting

To test the accuracy of our first generation automatic counting program we compared it to manual counting. Two tests were performed; first by seeding cells labeled with either green or red fluorescent dye onto two different screening chips; second by seeding three different chips with labeled cells in three ratios of red to green; after screening images were analyzed both manually and automatically. Overall, we found only small discrepancies between the two counting methods. We found that the accurate detection of wells containing cells was slightly lower for far red-DDAO labeled cells than for the calcein AM labeled cells, 97.4% and 99.7% respectively. The accuracy of determining the total numbers of cells were similar for both dyes, although it was evident that it decreased with increasing cell density. This probably reflects that our software, although good at distinguishing single cells from clusters, was not as good at counting the number of cells inside clusters as the human eye is. In most experiments the cells are seeded as single cells and form clusters later. The images here are taken directly after seeding, which could partly account for the accuracy. The difference between the calcein and far red-DDAO is most likely correlated to the more homogenous calcein staining, giving higher accuracy of determining cell numbers.

We then compared our method to flow cytometry. This was done by mixing two populations of differently labeled cells (calcein AM and far red-DDAO) at different frequencies in different chips. Automatic counting of the total number of green and red cells in each image was performed with our software, which enabled determining the frequencies within the total population. The same mixtures were also analyzed with flow cytometry and the results were found to be comparable.
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Extensive evaluation of the second generation program has unfortunately still not been performed though the initial tests indicate results comparable to manual counting.

3.3 Population heterogeneity

One of the main goals when designing and evaluating the device was to characterize the heterogeneity within immune cell populations. In our first experiments the heterogeneity became apparent as it clearly manifested itself in the varying behavior of the cells. Even in the simplified studies (Paper I) we saw large variations in migrational behavior and cytotoxicity between individual NK cells. To further investigate these differences more controlled in depth studies are currently being performed (manuscript in preparation).

3.3.1 Cytotoxicity

The aim of developing this microchip-based assay was to be able to study the cytotoxicity of immune cells on the single cell level. At first we were mostly intrigued by the fact that NK cells showed such variation in the way they killed (Paper I). It also became apparent that it was not only the killing fashion that varied, but also the total cytotoxic potential. Therefore our subsequent studies (Paper IV, V, and manuscript in preparation) have become more focused towards describing and elucidating the mechanisms behind this variation.

3.3.1.1 Fast and Slow Killing

The most apparent difference regarding cytotoxicity was the difference the dying cells displayed when hit by an NK cell. Two types of events were detected and quantified; they were denoted fast killing and slow killing. This difference was first observed in (Paper I) and then further evaluated in (Paper IV). Fast killing was visibly identified as an abrupt rupture of the target cell membrane causing the fluorescent viability dye to immediately leak out from the dying cell. In paper I, it was defined as an uninterrupted initial intensity drop larger than 75 % with no or insignificant further leakage. Slow killing was almost always also initialized by an intensity drop, but significantly smaller than 75 %, and then followed by slower leakage. The initial intensity drop is thought to come from when the perforin first pierce through the membrane, forming channels to subsequently let the NK cell deliver its cytolytic granules into the target cell. The perforin channels make the target cell membrane momentarily leaky and some of the dye will escape. Interestingly, about half of the targets cells killed by slow killing also display membrane blebbing, while cells with abrupt calcein leaking showed no blebbing but instead rapid swelling and bursting. In total, fast killing made up approximately 30 % of all the killing events characterized in Paper I (n=117). In Paper IV we also studied the previously introduced concept of fast and slow death - although the definition of fast vs. slow was slightly refined. Here approximately 35% of the targets were killed by fast and 65% by slow target cell
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death, this corresponds well to the data in Paper I. In both papers the 293T targets were used.

It is possible that these distinct patterns could reflect two mechanisms of NK-mediated target cell death or different killing efficiency of individual NK cells. However, we found no correlation between the conjugation time before initial calcein leakage and the type of death induced in Paper I, while in Paper IV the mean time to lytic hit was approximately 3 times faster for fast death, although there was a large spread of values making the difference ‘non significant’. Another interesting feature is that only about 40% of the NK cells making contact with targets displayed cytotoxicity in Paper I despite the fact that all target cells NK susceptible tumor cells (Paper I). In Paper IV the level of cytotoxic NK cells was calculated to 65% (n=140) out of the ones that did interact with target cells. Interestingly both of these levels are consistent with the screening results for 293T target cells in Paper V, where some preliminary results indicate a donor specific difference for the NK cells; either an activity level around 40% or around 60%.

![Figure 6](image)

**Figure 6. Times used for characterization of NK-target cell interaction.** Target cell is shown in green and NK cell in blue. The times that were investigated were as follows: 1) Time to lytic hit, 2) time from lytic hit to visible death of target, 3) conjugation time, and 4) attachment time. Picture adapted from Paper IV.

In paper IV, in depth characterization of the cytotoxic response of individual NK cells have been investigated. Here we defined 4 different times that we used to characterize the interaction between NK and target cell: the conjugation times, attachment time, time to lytic hit, and the time from lytic hit to visible signs of target cell death (Figure 6). This together with the number of individual target cell conjugations was analyzed for a great number of NK cells. This data has then been used to e.g. look at the correlation between speed and
strength of the NK response and to categorize NK cells based on their contact/killing history.

In this study we found that NK cells spend less time in conjugation (time 3) when in lytic compared to non-lytic interactions. Also it was apparent that target cells sometimes have difficulties to detach from the NK cell after periods of stable conjugation, present as long attachment times (time 4). We could also see that delivery of a visible lytic hit, detected as an intensity drop, was indeed lethal, 99% of the target cells that received a lytic hit later showed signs of visible death. Death was also induced fast, generally occurring within 60 minutes after the lytic hit (93%).

One important finding regarding the killing and conjugation fashion was that both mean conjugation and attachment times were shorter for interactions eventually leading to fast target cell death: mean conjugation fast (≈20 min) vs. slow death (≈90 min); and mean attachment fast (≈90 min) vs. slow death (≈180 min). For non-lytic interactions the conjugation times varied, bearing more resemblance to slow death for the conjugation time and more like fast death for attachment time. Also, as discussed before, the mean time an NK needed to make the decision to deliver a lytic hit was shorter in the fast killing interactions (Paper IV, Fig.4B-D). Clearly in this study there was a correlation between fast killing and relative speed (shorter time spent for target-cell interaction), of the NK cell. However, what became increasingly clear to us was that the difference between individual NK cells are very large emphasizing the importance of single-cell studies to appreciate the full range of immune cell behavior. Also, from a technical point of view, both the migration and screening wells worked very well to retrieve large amount of data over a long period of time about individual NK cell behavior. This was possible because the microwells allowed imaging the same cells continuously without loosing them from the field-of-view.

### 3.3.1.2 Target cell and Donor specificity

In all studies evaluating cytotoxicity in this thesis primary human NK cells are challenged with either 293T or K562 target cells, which are both efficiently killed by IL-2 activated NK cells. K562 cells are MHC I deficient, whereas 293T cells express low levels of MHC I (mainly HLA-E, but also other types of MHC I). In paper V we compared the cytotoxicity towards the two cell lines. We observed a slightly higher cytotoxic potential against K562 targets for the same NK cell donor (unfortunately only one observation) and also the average active NK population increased slightly when charged with K562 cells (Figure 7A). This is in line with the observation that NK cells in general seem to start killing K562 target cells faster than 293T targets (unpublished data). Another interesting observation is that NK cells may be more specific in their cytotoxic response against 293T. Most of the NK cells that continued to kill 293T targets cells after 6 hours killed were NKs that had already killed in between 0-6 hours, whereas for killing of the K562 the number of new and old killers were more or less the same (Fig. 7B).
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Figure 7. Cytotoxicity screen of IL-2 activated NK cells challenged with two target cell lines. A) Fraction of the NK cells that had displayed cytotoxicity after 6 and 12 hours towards 293T cells (4 individual donors), and K562 cells (3 individual donors) in black, mean value in red. Spontaneous target cell death during the same time is shown in grey. One donor’s NK cells were tested against both target cell lines, these data points are connected by the grey dotted arrow. B) Percentage of ‘new’ and ‘old’ killers between 6-12 hours. Old killers were NKs that had already killed in between 0-6 hours and new killers ones that were previously inactive. The majority of NK cells that killed 293T target cells were ones that continued to be active, whereas for killing of the K562 the number of new and old killers was more or less the same. Picture adapted from Paper V, Figure 2.

For all studies presented in this thesis the genotype of the NK cell donor was unknown. From what we have seen the difference between individual donors seem to be smaller when it comes to killing of the MHC I-deficient target cell line K562, than for the 293T target. We have observed some individual differences for the 293T cells that we believe may be donor depending. In Paper I approximately 60% of the NK cells that made contact with 293T target cells did not kill. In Paper IV, Figure 6C, this percentage can be calculated to 36%. In Paper V we looked slightly more on the individual donor level and can make out 2 possibly distinct subgroups, where 2 donors kill the 293T targets more efficiently than the other two donors; 50-60% compared to 30-40% active killers (Fig. 7A and Paper V). We have previously seen that the NK cells from a single donor perform equally well on day 5-9 after IL-2 activation (unpublished data), so all data in Paper V is obtained in this timespan to rule out other differences.

To draw any clear conclusions from this is however not possible because only 4 donors were investigated in parallel, but interestingly this data is in line with the data presented in Paper I and IV, and these donor specific differences is something that we would like to investigate further. This could for example be done by genotyping the donors before screening and see if they express any different KIRs, or other important activating or inhibiting receptors. A plausible reason for this difference is that some donors’ NK cells are slightly inhibited by the MHC I expression of the 293T cells, e.g. if the NK express high levels of NKG2A or corresponding KIRs. On the other hand, for the K562 target cells the
‘missing self’ recognition may be the main type of activating signal perceived by the NK cell. To further investigate this a more controlled system like the mouse system would be attractive. However, mouse NK cells have been tried out for other studies within the microwell platform, and so far not worked well because of their low activity \textit{in vitro}.

None of these studies have been extensively enough to draw any clear conclusions, however these are all intriguing observations that may serve as an inspiration for designing future experiments.

### 3.3.1.3 NK serial killing

The phenomena of serial killing have previously been described in the literature, both for T cells\textsuperscript{113, 114} and NK cells\textsuperscript{115, 116}. In Paper IV we saw there was a significant heterogeneity within the NK cell population; both regarding the number of targets killed, with some NK cells killing up to 7 targets within 12 hours; and the consecutive fashion in which the NKs killed.

There have been some different definitions used in regards to serial killing NK cells. When they were first described in 2007\textsuperscript{116}, they were described as NK cells consecutively killing multiple target cells. In our first study NK cells killing 5 or more targets were defined as serial killers (Paper IV), but in another recent paper by Christakou et.al the more general definition (also used for humans) of 3 or more kills were used\textsuperscript{97, 117}. In our most current study we have chosen to stick to this definition. In Paper IV we also saw that serial killing NK cells were larger compared non-serial killing NK cells. However, size in this study was based on the mean area of the NK cells during the whole experiment, which will be effected by the migratory behavior. A small but elongated cell will appear larger than it actually is. In addition, we only compared killers to serial killers, and the non-killers were not considered. Therefore, we later aimed towards elucidating these features more thoroughly in a study devoted to investigate the morphology and the expression pattern of cell surface receptor of these highly cytotoxic cells (manuscript in preparation). A few of the findings will be discussed here.

The first features that were investigated were the migratory phenotype and the relative volume of the NK cells, which was then related to number of target cells killed. This analysis was performed post-screening on all single NK cells that fulfilled a set of requirements: 1) they resided in wells where there were still live target cells left to be killed at T\textsubscript{6h}, so that lack of targets is not the reason they stopped killing; 2) did not divide or die in between 0-6 h; 3) and were not out-of-focus or in too close proximity to the well wall to affect the measurements. If these prerequisites were fulfilled the image from T\textsubscript{0} were used to measure mean intensity, area, and perimeter of the NK cell (Fig. 8A). These values were used to calculate the total intensity, which can be assumed to be roughly proportional to the volume, and the roundness of the cell.
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Figure 8. Serial killing NK cells are larger and have a more migratory phenotype.
A) Example image from a cytotoxicity screen at T₀. Calcein and DDAO stained 293T target cells are scored as alive (green/yellow) or dead (red), NK cells are shown in blue. From this type of image the individual NK cell's total intensity and roundness value are measured, a perfect circle has a roundness value of 1.0. B-C) A larger fraction of the highly cytotoxic NK cells were both larger and had a more elongated phenotype (54.1%) compared to the moderate killers (23.7%) and non-killers (14.5%). Total intensity is assumed to be proportional to the volume and a small roundness value indicates a more migratory phenotype. Non-killers (green bars), killers (orange bars) and serial killers (red bars).
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First an estimate of the relative NK cell volume was calculated as follows. All individual total intensity values were normalized to the mean total intensity value of all the NK cells in that particular experiment. This was done to compensate for differences in staining and laser settings between different experiments. Therefore normalized total intensity value indicates whether the NK cell has a smaller (<1) or larger (>1) volume than the average NK cell in that experiment. Then we approximated the roundness value, which can be seen as a measurement of the shape of the NK cell, although this is only a snap-shot image. The roundness value varies between 0-1, where 1 is a perfect circle. Hence, a cell with a highly migratory phenotype will have a small roundness value.

\[
\text{Roundness} = \frac{4 \pi \times \text{Area}}{\text{Perimeter}^2}
\]

These two values were then displayed in a scatter plot for all the 3 categories of NK cells we have defined: non-killers, killers and serial killers. We find the large cells with highly migratory phenotype in the upper left (UL) quadrant (Fig. 8C). Interestingly we find there that higher percentage of serial killers in the UL (54,1%) compared to killers (23,7%) and non-killers (14,5%). This data fits well with previous findings, but here we can see that on average a serial killing NK cells are both larger and have more irregular shape. It is promising for the method that the phenotype is so distinct that it is evident enough to be interpreted from only a snapshot image (although there is of course great individual variation and some cells may not display their general phenotype at the particular moment the image was taken). One interesting observation regarding this is that in fact most NK cells stop migrating when conjugating with target cells; this usually means their roundness will increase. Approximately 60% of the serial killers are in conjugates at T₀, compared to ≈20% for the non-killers and ≈40% of the moderate killers. Despite more NK-target conjugates for the serial killers they end up with a more migratory phenotype.

To further investigate the phenotype of the serial killer we were interested in their expression phenotype for some activating receptors. In order to test this hypothesis we planned to add an additional antibody-staining step to the general screening. Immediately after the finished screen the whole chip with the NK cells would be stained with fluorescently tagged antibodies specific for the receptor of interest, and subsequently an extra set of images taken. We believe that staining after the actual assay has the advantage of not tampering with the cells beforehand, which may have unknown side effects on their behavior. The first step was to test whether antibody labeling of cells within wells was feasible at all. Our main concern was that the fluorescent background levels would occlude the signal due to inefficient washing of antibody after staining. This was especially true for some rare receptors, as they were expected to have a very weak signal due to low expression levels. Fortunately, this was not the case for
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Figure 9. Antibody staining of cells inside the wells. The first tests of antibody staining was performed with only newly seeded unstained NK cells as proof-of-concept that the antibodies could actually reach the cells at the bottom of the wells and subsequently be efficiently washed to acquire nice staining. Here we used anti-human LFA-1 Alexa 488 antibody and washed the chip twice. Imaging was done with a 20x objective.

Our initial labeling experiments where we acquired high-quality images of live NK cells stained with anti-LFA-1 (Fig. 9).

Once the antibody-staining protocol for microwells was established we started to try it out on real screening experiments. However, so far we have not been successful in obtaining this type of data so this approach remains to be further evaluated in future work. It is an alluring thought – to be able to link the actual expression levels of e.g. DNAM-1, NKp46, NKG2D, NKG2A, or other candidate receptors to the serial killing of individual NK cells.

3.3.2 Migration behavior

We found large variations in the migrational behavior of individual NK cells. This was evident already from our first cytotoxic studies using time-lapse in small wells (Paper 1), but not thoroughly characterized. Here the difference in migrational behavior only influenced the time-to-first collision, which was characterized and compared to the simulated cells. A small subset of cells did not move at all and hence never collided with any target cell, this was mirrored in the less than 100 percent collision-rate even after as long as 9 hours incubation.
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This feature was more thoroughly evaluated in our larger migration wells (Paper IV), where they were used for studying heterogeneity within the same population. Here the free migration, cell-cell contact patterns and average conjugation times of single NK cells were studied. However, this paper’s main focus was characterization of the lytic response pattern in depth and not the free migration periods.

Two studies performed by other members of the lab have focused more towards the actual migrational pattern, both free migration and in conjugation with target cells. The first study analyzes the relative importance of environmental activating factors such as available targets, by comparing IL-2 activated primary human NK cells migrating in the presence or absence of target cells. Here the heterogeneity in migrational behavior was apparent as both the mean speed and the time spent in directed compared to random movement varied a lot between cells in both groups. The second, more recent study, has compared the migrational and cytotoxic behavior between unactivated NKs and NK cells preactivated with IL-2. In this study, average migration speeds were found to be similar, but activated NK cells had a more dynamic migration pattern, as they changed more frequently between low and high grade of motility.

All studies mentioned confirm the idea that there is a large heterogeneity in migrational behavior within populations as well as between differently preconditioned populations. This notion can have impact when it comes to for example preconditioning of NK cells for adoptive transfer.
4. **Discussion and Future Perspectives**

In this project we have successfully developed and evaluated a microwell chip platform for screening of individual immune cells. We have then used it for studying the cytotoxic behavior of NK cells but also to a lesser extent T cells. Both of these types of immune cells have a potential value for medical applications for which our platform can also be useful. I will therefore start this concluding section by introducing the type of additional applications it can be used for; both future cytotoxicity studies in general and medical applications. Then I will go on to a more general discussion regarding the results we have obtained so far.

#### 4.1 Applications

Single cell assays can be used to answer a variety of biological questions, of both scientific and medical importance. I will here discuss three of the applications that the microwells were initially designed for, some of which we are currently working on, and their potential use we foresee for the future. They are by no means limited to these applications, but rather versatile tools when working with any cell population.

##### 4.1.1 Cytotoxicity studies

At the beginning of this project many basic biological questions regarding NK cell cytotoxicity still remained unanswered because of the intrinsic difficulties of monitoring motile cells for extended periods of time, and most of the previous studies had been done with FACS or microscopy methods that do not allow long-term imaging. Although we have now successfully used our microwell platform to better understand some aspects regarding NK cell biology, the heterogeneity of NK cells is far from understood. For example, it would also be interesting to quantify the distribution of inactive to very active NK cells and see how they differ in expression levels of various activating and inhibitory receptors. There are also other basic questions that still need to be addressed; if an NK cell becomes exhausted after a certain number of killing events, what is then the ultimate outcome? Will it undergo apoptosis or maybe even start proliferating? Does a serial killing NK cell not only kill its target faster (Paper IV), does it also migrate faster hence encountering more targets? By exploring the versatility of the platform different imaging strategies can now be used to answer these types of questions.

The larger wells are optimized for monitoring both migrational behavior and cytotoxicity of individual NK cells. Tracking of individual NK cells over time will gain a huge amount of information regarding migrational behavior, cell-cell contacts and their outcome. However, these wells need to be continuously monitored to keep track of the cells; therefore the possible imaging time may be limited. The screening wells can instead be used for less complicated and time-
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consuming analysis of cytotoxic behavior. By imaging the wells at only two or a few timepoints we lose dynamic information of e.g. cell-cell conjugation time but gain power in the number of cells that can be analyzed. Trapping of single NK cells together with a few target cells is advantageous because we maximize the biological information we gain, while minimizing the assay time by increasing the chance of cell-cell encounter. Since NK and target cells are trapped in wells, quantification of cytotoxic potential and late outcome like proliferation or apoptosis becomes feasible for large numbers of cells. These types of studies may also be performed on different subsets of NK cells and ultimately correlate functionality to expression of surface receptor or educational background.

4.1.2 Stem cell transplantation
As discussed in the section about HSCT it has become clear that only a small number of cells are responsible for causing GVHD in post-transplant patients. Finding a reliable way of testing for possible occurrence of this condition and its predicted severity beforehand would greatly improve the odds for these patients. Because of the very small number of cells being responsible, testing with conventional methods has so far been impossible.

Here we propose a method where we take advantage of the long-term cell culturing properties of our multiwell screening chips. By incubating donor T-cells together with recipient peripheral blood mononuclear cells (PBMCs) and detect T cells attacking the recipient. Approximately only a few percent of the T cells are alloreactive so detection of these rare events is only possible by looking at a great number of interactions, which is in theory feasible with our screening approach. Because of the large amount of wells the eventual read-out will have to be performed by automatic counting of dead and living cells for each time-points coupled to a subsequent statistical analysis. This approach would be fast and require small patient samples.

A fairly new focus within the transplantation field is how the NK cell repertoire of the donor and recipient will effect the outcome. In one of the first studies of this they saw that the donor NK cell-alloreactivity not only protected the transplanted patient from GVHD and graft rejection, it also had the potential to eliminate leukemia relapse. Therefore, new strategies where our screening platform is used to match the donor and recipient based on NK cell reactivity would potentially be valuable.

4.1.3 Clonal expansion and adoptive transfer
Clonal expansion is a feature common to all adaptive immune responses. When a naïve T or B cell recognizes its antigen on an activated APC it stops migrating and enlarges - the cell becomes a lymphoblast. The lymphoblast then begins to proliferate vigorously, dividing two to four times every 24 hours for 3-5 days, so that one naïve lymphocyte can give rise to around 1000 identical daughter cells. These cells then differentiate into effector cells.
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Clonally expanded T cells (or NK cells) are of interest to use for adoptive transfer in treatment of e.g. cancer or certain viral infections\textsuperscript{123}. This can either be done by transferring the patient’s own cells after specific expansion or by allogeneic transplantation of selected lymphocytes. This has been especially interesting in a posttransplant setting when the patient often is affected by viral infections because of the necessary myeloablative pretreatment\textsuperscript{124, 125}. T cell depletion performed on the donor infusion have also been shown successful to limit acute and chronic GVHD\textsuperscript{126}. However, this did not translate into improve overall survival rates, mainly because of Epstein-Barr virus–associated lymphoproliferative disorders\textsuperscript{127}, and disease recurrence. Strategies to avoid GVHD and at the same time protect the patient from viral infections during engraftment of the new immune system are therefore desired.

By only infusing the patient with cells specific for one or a number of pre-selected peptide antigens, one avoid infusing potential alloreactive T cells that might attack the patient and cause GVHD. Many methods to accomplish this have been suggested, e.g. selecting cells with peptide-MHC tetra- or pentamers\textsuperscript{124, 128, 129} or cytokine-capturing methods\textsuperscript{130}. Peptide-MHC complexes are used to capture antigen-specific T cells since they bind to the presented antigen. The complex, together with the bound cells, can then be fished out by e.g. FACS if they are fluorescently labeled. Subsequent culturing of the selected cells may also be required depending on the protocol, slowing down the process and ultimately often making it too late to save the patient. However, without further culturing the T cells are still bound to the tetra- or pentamers which have an unknown effect when injected into the patient\textsuperscript{129}. Another disadvantage of this method is that the peptide sequence is required and peptides need to be synthesized; when the peptide is unknown the method cannot be used. Cytokine-capturing methods have the disadvantage of possibly exhausting the cell during selection, hence making the subsequent adoptive transfer useless.

A chip based selection method would have the advantage of a polyclonal response where the cells are untouched and can be directly injected into the patient after a short expansion. Because the selection is polyclonal we start out with a larger population which decreases needed time for proliferation to obtain the crucial amount of cells for transfer. In the T cell setting it is also possible to transfer both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, which may increase the survival of transferred cells by natural proliferative signals. Also, knowing the exact antigen is not needed which is necessary for using the peptide-MHC complexes.

Cell therapy using NK cells is also possible, but here the donor’s alloreactive NK cells are beneficial in the sense that they will kill host target cells, in particular leukemia cells. The alloreactivity is caused by the missing expression of KIR ligands on the recipient’s cells\textsuperscript{131-133}. Because the donor and recipient express different MHC I alleles, the recipient MHC I will not be able to block all donor NK cells. In one study, cell therapy using NK cells from haploidentical donors used in clinical trials was evaluated. Here the degree of
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Incompatibility of KIR ligand was closely correlated to evidence of NK cell clones killing recipient target cells, and the number of alloreactive NK cells in the graft was correlated to patient overall survival\textsuperscript{121}. In an adoptive transfer setting our platform may be useful for testing the activity of individual donor NK cells towards both recipient tumor cells and healthy cells. One can then select donor based on the level of alloreactivity to maximize the chance for the patient. It may also be useful for selecting and clonally expand e.g. the more potent serial killing NK cells and use those for adoptive transfer. Another potentially beneficial study is to test how different expansion protocols actually affects the cytotoxicity of the NK cells - in the end the total number of NK cells you put in the patient is not what counts, it is their total activity that can make a difference.

4.2 Screening and the microwell platform

We have evaluated two different microwell systems, one in PDMS and one in silicon. Overall, the silicon chips were found to be more practical for our studies compared to the PDMS chips. First of all we discovered that the PDMS wells were not deep enough to keep cells confined and therefore required an additional sealing procedure. This proved technically difficult with unreliable cellular distribution across the chip and relatively short-term cell survival. Deeper PDMS wells may solve the confinement issue, but fabrication of such structures poses new challenges as the delicate soft chip need to stay intact after being peeled of the master. In addition, imaging in deep uncovered PDMS wells need to be conducted through a bottom layer of PDMS preventing high-resolution imaging. The silicon chips, on the other hand, were easy to handle, gave reproducible distributions of cells, allowed cells to proliferate normally, and were compatible with long-term high-resolution imaging (as cells are imaged through a normal cover glass).

By simulating the migrational behavior for different types of cells in wells, we tested the potential benefit of scaling down the interaction area for rapid screening approaches (Paper I). We not only saw a clear correspondence between the simulated well size and the mean time to cell-cell collision, which was expected, but the results were further reflected in the biological experiments showing that our model of cell migration was accurate. Even if two cells are confined in a relatively small well it may take some time for them to meet. For example, roughly 50 percent of the primary NK cells encountered a target cell within 1 hour both experimentally and in our simulation (Paper I). However, we did observe slight differences between the simulations and the experiments mostly due to that all NK cells were considered motile in the simulations while some real NK cells were found to be temporarily or completely immotile. Therefore not all NK cells encountered a target cell in the duration of the experiment even when using small wells. However, this was quite uncommon. For long incubation times (>12 hours) it is therefore still reasonable to assume that two cells in a well have been in contact with each other during
the incubation period. This is the idea behind a two (or a few) time-point screening approach where the incubation time can be varied depending on the set up. Taken together, the data presented in Paper I showed that our 50 μm silicon wells were indeed suitable for our screening approach.

What also need to be addressed are the smallest subpopulations that are possible to detect with the cytotoxicity assay. The seeding procedure used today is still based on random sedimentation into the wells, with the advantage of being easy to perform but it is uncontrolled. There is always a risk that some cells end up alone in wells and naturally are prohibited from interacting with other cells – this is irrelevant when it comes to target cells, but may pose a problem when we want to look at very small populations of effector cells as these cells can be missed. One way to reduce this problem could be to increase the number of seeded target cells, but there is no way to completely avoid empty wells and still not overfill the rest. The wells with only target cells instead supply us with information about the spontaneous death rate, which should be low in successful experiments to rule out other inflicting factors than effector cell cytotoxicity in the majority of cases (Paper V). At the moment no efficient techniques are available for seeding living cells into such small wells in a controlled manner, but if this becomes available in the future accuracy may be improved. A variation of techniques have been suggested suitable when scaled down, either similar to those used for printing of protein arrays or for FACS sorting. Microarray printing heads are constantly coming down in size but this method is still not very gentle to the cells and up to 10 % may be accidently lysed during seeding \textsuperscript{134, 135}. Scaled down FACS sorting is therefore probably a better strategy in the long run, especially as it can be combined with gathering information of the cells being sorted if gated on various receptors.

Up till now very few studies have been devoted to high through-put studies of immune cells on the single cell level, but they are increasing in number (see for example \textsuperscript{136-138}). One exception is a recently described method used for monitoring target cells being killed by CTLs where individual cells are trapped in small funnel-shaped wells for up to 24 hours \textsuperscript{87}. However, this approach is more limited than ours, as it does not enable high-resolution imaging, quantification of multiple killing events, migration behavior or studies extending several days. What is still interesting is that they observed that target cells could be killed either rapidly or more slowly, and propose the possibility of alternate killing mechanisms. The similarity to our results for NK cells are striking and maybe this overlap reflects processes that are common for NK cells and cytotoxic T cells.
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4.3 Analysis

An automated analysis program is a prerequisite for efficient screening of a full microwell chip, and first we showed that our first generation automatic counting program only gave rather small discrepancies compared to manual counting (Paper II). During the course of the study development of this program has been necessary due to more challenging images. This has resulted in our completely new approach in the second generation program. Though still in its infancy and in need of more thorough evaluation, it has solved some of the intrinsic difficulties regarding the handling present in the first generation program. Manual counting may always be more accurate, especially when it comes counting the number of cells in clusters, but we do solve the problem of bias by implementing a program as it treats all data sets the same. Also, it is possible that the new program will have larger difficulties with other target cell lines than K562, which has a very round morphology, due to algorithm used. Usually 293T targets have a spherical phenotype upon seeding, but as they attach to the glass they often display a more elongated triangular shape. Also, the NK cells themselves often display an elongated and migratory phenotype, especially the highly cytotoxic ones as discussed before.

Both automatic counting programs are dependent on stable and homogenous fluorescent staining; most importantly all cells need to be stained and preferably to uniform intensity. The staining process usually works very well, thus the one remaining challenge for optimizing image quality is the focus issue. This needs to be solved in order to achieve optimal accuracy from the automatic analysis of a full-chip screen.

Naturally, because a lot of time has been spent on performing manual analysis of screening results, I find it promising for future studies to have this new tool available. However, without manual analysis evaluation of the automatic programs would have been impossible, as we need to know what is ‘correct’ in order to say that our program performs well enough. Also, the need for manual analysis has not completely vanished. Maybe a combination of both will in the end always be needed for some applications. When performing manual analyses of dead or live cells we have identified some features of the targets or NKs that are potentially hard to handle for the automatic counting – some of them are cell type specific others more general: NK cells tend to cluster all the dead target cells into a pile. This goes with the finding in Paper IV that often they have a hard time to detach after periods of stable conjugation. Therefore they tend to pull their old already killed targets along when killing new targets. Possibly because they are not operating within a matrix as they would in vivo, hence there is no force retaining the target. It can be challenging to count piled up cells accurately, especially for a program. In this case it would be valuable to have the program extract the difficult wells so that they can be manually analyzed, but still use the automatic program for all the other wells to save time. Generally the most efficient serial killers may end up
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in the ‘difficult wells’ as they also tend to collect their victims and they are naturally surrounded by more dead targets.

Automatic counting will always be most interesting when we need to analyze really large amount of data, as is the case for e.g. the screen for GVHD. Then we do not need to count the exact number of killed target cells very accurately, it is more of a binary calculation – active vs. non-active T cell. Here it is absolutely crucial to have a program in order for this type of analysis to even be feasible in theory.

Another question regarding the analysis of screening results is related to the interpretation of final data. Because we do not follow all the events over time some information will always be lacking – but how can we chose to handle this to introduce the least error into our analysis? This is something we are well aware of and there are different strategies to tackle this. The main question is whether one should ascribe all the dead target cells within a well at T₆h (including targets that were either dead-seeded or killed before imaging at T₀h) to NK cell cytotoxicity or only count the cells that were definitely killed between 0-6 h? The intrinsic problem is that unless you ascribe all the kills at T₆h to the NK cell you will loose valuable data, but you will also erroneously label a few NK cells as killers although they are not. For the NK cell screening experiments killing of K562 targets is initiated very fast after seeding. This becomes an issue especially for identifying and characterizing serial killers - those NK cells are highly cytotoxic and may already have killed as many as three or more targets at T₀ (unpublished data). When analyzing this type of data it seems more reasonable to include all dead target cells, mainly because the majority of cells that will erroneously become killers are single-target-cell killers. We are mostly interested in the ones killing three or more. An alternative way would be to perform statistical analysis to calculate the probabilities that the NK is the culprit. However, this would require more advanced analysis and may be a way to enhance experimental reliability in the future.

The influence of dead-seeding increases with low activity of the effector cells, e.g. the not very cytotoxic T cell clones used in Paper V. In this study, there were also quite a high number of dead-seeded target cells, but fortunately very low spontaneous target cell death. Although we may miss some active cells, the only feasible mean of measuring cytotoxicity here is to count the number of target cells that actually did die in between 0-6 h. This strategy eradicates the problem of ascribing cytotoxicity to cells that in fact did not kill.

This is a dilemma one has to think of when analyzing this type of screening data; therefore the information we get from T₀ is indeed valuable to decide which method will introduce the least error. For time-lapse this is not an issue since the imaged area is smaller and you can follow the NK cell and not just interpret the snapshot outcome. Despite this, we believe the benefits of the powerful screening tool are enough reason to use it, as long as we are aware of its limitations. The promise of gathering data from such large number of events
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weigh-up for the small errors introduced, enabling studies of rare phenomena that would otherwise not be possible.

4.4 Serial killing

Naturally this thesis have dealt a lot on the issue of serial killing NK cells as the development of the screening platform for investigating these has been the main focus of my work. Unfortunately part of our recent findings has not yet been fully evaluated, but some of the preliminary results are presented here.

Serial killing behavior was seen already during our initial cytotoxicity studies (Paper I), though it was not evaluated. It was later investigated in more depth both in the migration wells (Paper IV) and screening wells (paper V and manuscript in preparation). In Paper IV it was first noticed that serial killing NK cells were larger than the non-serial killers, and later been confirmed in more recent findings (manuscript in preparation) \(^{139}\). This goes well with the findings that IL-2 activated NK cells in general were approximately twice as big as the resting cells, displayed a more migratory phenotype, and were more likely to employ “motile scanning” of the target cell surface during conjugation \(^{118}\).

We now know that there are large heterogeneities in the total cytotoxic activity as well as dynamic parameters like conjugation times, fast and slow killing, etc. It seems that serial killing NK cells are engaged in more fast than slow killing events and also have generally shorter conjugation and attachment times compared to normal killers (Paper IV).

However, the long-term response of individual NK cells following contact with target cells is still largely unknown. It is still not clear if that leads to late NK responses such as anergy \(^{140}\), apoptosis \(^{141}\) or clonal expansion \(^{142}\). This question has remained unresolved but may now be one step closer to being investigated with the screening approach. Because the NK cells are only labeled with a cytosolic dye it has been challenging to accurately determine the fate of the NK cell itself. When they are alive and visible their number is easily scored, but when they are not present at some timepoint it is unknown whether this is because they are dead or if they momentarily migrated out of focus – which some of them do for shorter periods of time. This is generally not an issue for the time-lapse experiments where you can actually see the NK cell die or proliferate, but in the screening approach we only get snapshot images so we do not know what happen in between. Sometimes it is obvious from the transmission DIC image that the NK cell died, but in a well with a lot of remains from killed target cells this can be tricky. This type of analysis also become more challenging at later time points and requires very good initial staining of the NK cells. In our most recent study we have excellent staining so here we aim to evaluate this. What actually happens to the NK cells after killing or non-killing interactions with target cells is of course something that is very interesting to us. This may help us to answer some additional very important questions that relating to their potential usage in for example adoptive cell transfer.
5 Concluding Remarks

In this thesis we have shown that our silicon microwell platform is a versatile tool suitable for long-term, parallelized, high-resolution imaging of immune cell interactions. We have also proved that both the screening and migration approach work well for analyzing cytotoxic behavior of individual NK cells. Up till now we have designed, evaluated and used the platform for studies of cytotoxic heterogeneity in human NK cell populations.

Using the platform we have observed and described the concept of fast and slow killing for NK cells. Which bears a striking similarity to results presented for T cells\textsuperscript{87}, hence maybe this overlap reflects processes that are common for NK cells and cytotoxic T cells. Though they represent different branches of the immune system they share many features.

It has also become apparent that there are large individual differences regarding dynamic behavior such as time spent in conjugation with target cells, the type of induced target cell-death, and the number of target cells killed. In these experiments we have detected subsets of more or less cytotoxic cells and described them morphologically. What still needs to be addressed is how these cells differ from each other, on a receptor or educational basis. Initial work in that direction has started but not resulted in any relevant biological data yet. So far we have managed to perform antibody staining within the microwells, but not in subsequent combination with screening. This will be the next step towards obtaining really interesting biological data, enabling us to theoretically relate specific functional behavior to an effector’s receptor repertoire.

Within the NK cell community it has become increasingly clear that NK cell repertoires are indeed highly heterogeneous and that the quality and quantity of an individual’s NK cell response depends on the MHC class I background \textsuperscript{143, 144}. The work in this thesis has helped to reinforce this view and emphasize the importance of single-cell assays to investigate the full range of behaviors that population-based assays cannot. Therefore we continuously aim to assess even more features of NK cell heterogeneity with our method, and ultimately to address questions related to NK cell education and tolerance. We are also interested in the more late responses of the NK cell itself, both from a biological interest point-of-view and because this is an important question in relation to these cells’ potential use in adoptive transfer settings.

Though we are well aware of the limitations of our screening set-up, only providing us with a snapshot outcome, we believe power in numbers will out-compete most of these issues. As long as we handle our data within its limitations the statistical benefits will be huge and we gain access to studying rare phenomena that would otherwise not be possible. With this platform at our hands we foresee an exciting future of better understanding and medical usage of these remarkable cells.
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