Ultrasound-assisted Interactions of Natural Killer Cells with Cancer Cells and Solid Tumors

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Abstract

In this Thesis, we have developed a microtechnology-based method for culturing and visualizing high numbers of individual cells and cell-cell interactions over extended periods of time. The foundation of the device is a silicon-glass multiwell microplate (also referred as microchip) directly compatible with fluorescence microscopy. The initial microchip design involved thousands of square wells of sizes up to 80 µm, for screening large numbers of cell-cell interactions at the single cell level. Biocompatibility and confinement tests proved the feasibility of the idea, and further investigation showed the conservation of immune cellular processes within the wells. Although the system is very reliable for screening, limitations related to synchronization of the interaction events, and the inability to maintain conjugations for long time periods, led to the development of a novel ultrasonic manipulation multiwell microdevice.

The main components of the ultrasonic device is a 100-well silicon-glass microchip and an ultrasonic transducer. The transducer is used for ultrasonic actuation on the chip with a frequency causing half-wave resonances in each of the wells (2.0-2.5 MHz for wells with sizes 300-350 µm). Therefore, cells in suspension are directed by acoustic radiation forces towards a pressure node formed in the center of each well. This method allows simultaneous aggregation of cells in all wells and sustains cells confined within a small area for long time periods (even up to several days).

The biological target of investigation in this Thesis is the natural killer (NK) cells and their functional properties. NK cells belong to the lymphatic group and they are important factors for host defense and immune regulation. They are characterized by the ability to interact with virus infected cells and cancer cells upon contact, and under suitable conditions they can induce target cell death. We have utilized the ultrasonic microdevice to induce NK-target cell interactions at the single cell level. Our results confirm a heterogeneity within IL-2 activated NK cell populations, with some cells being inactive, while others are capable to kill quickly and in a consecutive manner.

Furthermore, we have integrated the ultrasonic microdevice in a temperature regulation system that allows to actuate with high-voltage ultrasound, but still sustain the cell physiological temperature. Using this system we have been able to induce formation of up to 100 solid tumors (HepG2 cells) in parallel without using surface modification or hydrogels. Finally, we used the tumors as targets for investigating NK cells ability to infiltrate and kill solid tumors.

To summarize, a method is presented for investigating individual NK cell behavior against target cells and solid tumors. Although we have utilized our technique to investigate NK cells, there is no limitation of the target of
investigation. In the future, the device could be used for any type of cells where interactions at the single cell level can reveal critical information, but also to form solid tumors of primary cancer cells for toxicology studies.

**Keywords:** Natural killer cell, cytotoxicity, heterogeneity, multiwell microchip, microplate, biocompatibility, ultrasonic cell manipulation, 3D cell culture, solid tumor, spheroid, high-resolution imaging.
List of publications in the Thesis


VI. A.E. Christakou, M. Ohlin, B. Önfelt and M. Wiklund, “Ultrasound-assisted three-dimensional tumor formation in a multiwell microplate for monitoring natural killer cell functional behavior”, manuscript.

List of publications not included in the Thesis


**Author contributions to the papers**

I. Co-performed the trapping and viability experiments, co-performed the analysis of the viability experiments. Revised the manuscript.

II. Developed the first generation of chips. This involves, developing the protocols about cleaning of the silicon-glass multiwell chip, seeding and culturing cells in the multiwell chip. Designing and performing viability and proliferation experiments for three different cell lines. Designing and performing confocal imaging experiments of natural killer cell-mediated killing of target cells in the multiwell microchip. I revised the manuscript.

III. Performed the confocal imaging experiment for the different frequencies and frequency modulation.

IV. Designed and built the new generation wedge-transducer. Designed the holder and the structure of the updated device. I co-designed the experiments and I performed all experiments, except the NK cell purity tests, the 51Cr release cytotoxicity control tests and the high resolution imaging of the immune synapse experiment presented in Figure 3. I performed and analyzed all the measurements of the experiments except the cluster tracking analysis and I prepared most of the figures of the paper. I wrote a part of the paper and revised the manuscript.

V. Performed and analyzed the biological experiments and made the biological related figures. Wrote the part of the paper related to natural killer cells.

VI. Performed all cell cultures for the tumor formation experiments, co-designed the experiments and co-analyzed the data. Performed and analyzed all live cell imaging experiments of the tumors and the interactions with natural killer cells. Prepared the fluorescent imaging figures and wrote part of the paper.
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Chapter 1: Introduction

As a complex system the human body and its functions have been the center of exploration in order for scientists to answer fundamental questions about life and death. Despite the technological evolutions that has provided a great variety of tools for better decoding and understanding human nature, there are several areas that due to their complexity still remain relative unknown. One of these areas is the immune system.

“Though many lay unburied, birds and beasts would not touch them, or died after tasting them... The bodies of dying men lay one upon the other... [But] those who had recovered from the disease... had now no fear for themselves; for the same man was never attacked twice never at least fatally.”

Thucydides, History of the Peloponnesian War, 431–428 B.C.

The concept of immunity (from the Latin immunitas for “freedom from service”) had been recognized by the great historian Thucydides almost 2500 years ago.1

The immune system, a well-trained ‘army’ and major importance complex organization is composed of a great variety of specialized cells and molecules and it is responsible to repel harmful internal mutations and external invaders that can disorient the body from its natural prosperity and function. This ‘army’ is composed by different types of cells that each of them is assigned with a specific protective (or defensive) mission. However, within these subunits of immune cells that share the same responsibilities and characteristics, there is a functional heterogeneity that can be the key for decoding numerous incurable diseases. Such deceases although they have been unrevealed in some extend, still some mechanisms remain a mystery.

In order to explore the immune system and the heterogeneity within its population, we need to develop tools that focus on its core operation unit, the immune cell. Focusing on immune cells at the single cell level could facilitate decoding the individual performance against harmful substances, pathogens and malignancies that can cause disease.
In this Thesis, we have developed a microtechnology-based method for culturing and visualizing high numbers of individual natural killer (NK) cells interacting with cancer cells over extended periods of time. Thus, we can observe a heterogeneity with NK cell populations. The foundation of the device is a silicon-glass multiwell microplate directly compatible for fluorescence microscopy. The initial microchip (also referred as microplate) design involved thousands of square wells of sizes up to 80 µm, for screening large numbers of cell-cell interactions at the single cell level. Although the system is very reliable for screening, limitations related to synchronization of the interaction events, and the inability to maintain conjugations for long time periods, led to the development of the ultrasonic manipulation multiwell microdevice. With the ultrasonic microdevice we can induce simultaneous NK-cancer cell interactions by acoustic radiation forces generated in each of the wells of the microchip. Furthermore, we have utilized the ultrasonic method to induce formation of three-dimensional cell cultures, as solid tumor models to mimic in a more reliable way the in vivo conditions. Therefore, we can investigate infiltration properties of NK cells and their killing performance against solid tumors.

1.1 Immune surveillance

White blood cells (or leukocytes), substances produced by leukocytes and the complement system which is a large variety of plasma proteins with different antibacterial activities, comprise the immune system. A well-established and functional immune system require a sequence of actions. These actions involve the detection of a problem, the effective action that suppress the infection, the self-regulation and maintenance of the physiological state. Finally, they include an immunological memory comprised of long living cells that carry on information about the infection for a future immediate response against the specific pathogen.

The responses of the immune system are distinguished in two main categories, the innate and adaptive immunity regarding the rapidity and specialization of leukocytes against invaders and malignancies. Innate immunity is found in all animals and plants in different forms and is believed to exist throughout life evolution. The primitive nature and luck of specificity of innate immune system results to a rapid response that most often is effective enough to eliminate the pathogens usually before they escape immune protection and cause disease. However, in cases of unsuccessful handling of pathogens the innate immune cells can release a certain type of signal molecules called cytokines. Cytokines can trigger cells of the adaptive immune system to develop against of the specific pathogen. The adaptive immune system activation is slow comparing to the innate, nevertheless more efficient due to the high specificity of recognition, and action through sophisticated mechanisms that lead to rejection of the pathogen.
The biological target of investigation in this Thesis is the natural killer (NK) cell of the innate immune system and its functional properties. Although T lymphocytes are not used at any time in this work, I will explain shortly their characteristic recognition mechanisms since they are somewhat associated to NK cell recognition pathways.

### 1.1.1 Cytotoxic lymphocytes and T cell recognition

NK cells, T cells and B cells comprise the lymphatic group of the immune system and they have a critical role in host defense and immune regulation. T cells and B cells belong to the adaptive immune system and they are characterized by the high recognition and effector specificity against pathogens. In contrary, NK cells are considered to bear innate characteristics rather than adaptive due to the rapid response against targets without prior activation. However, this notion has been blurred by resent findings indicating adaptive properties of NK cells\(^3\)\(^-\)\(^6\).

NK cells and T cells are characterized as cytotoxic lymphocytes due to the exclusive killing mechanisms that they share against virus infected cell and cancer cells. T cells have the ability to recognize unhealthy cells through specific threat signals coming either from pathogens or mutated proteins. They can distinguish self-peptides from non-self-peptides (antigens) through a process called major histocompatibility complex (MHC)-restricted recognition, where the T cell receptors (TCR) bind to the bimolecular complex formed by MHC molecules and the antigen peptides\(^7\). TCR recognition together with co-stimulatory signals leads to T cell activation and proliferation.

After activation T cells can recognize the same peptide-MHC (pMHC) complex and either become cytotoxic T-lymphocytes (CTLs) that are ‘serial killers’ against virus infected cells expressing the specific antigen, or differentiate into CD4 T-helper cells. T-helper cells play a major role in immune regulation through secretion of cytokines that are responsible for activating B cell antibody production\(^2\),\(^8\). The effector T cells after proliferating and fighting the infection, decrease their number dramatically. The low numbers of cells that remain alive, differentiate into long-living cells called memory T cells that ensure a faster immune response against this virus if the infection occurs for the second time\(^9\),\(^10\).

### 1.1.2 Natural killer cells and immune surveillance

Natural killer cells were discovered in the 1970s and were initially thought to be an ‘experimental artefact’ in T cell cytotoxicity assays in mice, that revealed a subset capable to induce cytotoxicity without prior sensitization\(^11\),\(^12\). Human NK cells were at first characterized as non-adherent, non-phagocytic, large granular lymphocytes (LGL)\(^13\). Later it was shown that the phenotype of NK
cells could vary from LGL to normal sized lymphocytes depending on their activation level, thus limiting the detection possibilities based on morphology\textsuperscript{14}.

Besides morphology, also the recognition mechanism was initially unclear until the ‘missing self’ hypothesis was formulated in 1990 by the Klas Kärre group. This hypothesis suggested that NK cells choose the target according what is missing rather than what is expressed. They would attack cells that express low levels of MHC class I molecules due to an inability of the targets to engage MHC inhibitory receptors on the NK cells\textsuperscript{15}. This notion was dominating until a new model was proposed; that NK cells also express activation receptors that recognize specific viral antigens. And that the function of NK cells against tumors and virus-infected cells was regulated by a balance between activating and inhibitory signals after ligation of a wide range of receptors on NK cells\textsuperscript{16}.

According to this concept, the reason that NK cells do not always respond against cells that lack MHC surface proteins can be due to absence or insufficient activation signals. For instance, although human erythrocytes do not express MHC class I molecules, they are not targets for NK cells. This is due to the fact that healthy erythrocytes besides MHC Class I, they also lack ligands that bind NK activating receptors\textsuperscript{8}. This NK recognition mechanism also correlates with the fact that some viruses and mutated cells have evolved strategies to downregulate expression of MHC molecules on the cell surface in order to escape T cell recognition and T cell mediated cytotoxicity. But this viral activity will cause the absence of sufficient MHC molecules to inhibit NK cell activity and therefore NK cell are triggered against the targets\textsuperscript{17,18}.

Although NK cells and CTL functions are complementary and together comprise a powerful tool against infection, it is still possible for certain virus to avoid recognition and elimination by cytotoxic lymphocytes\textsuperscript{2}. An example is HIV virus that can balance between escaping from CTLs antigen specific recognition and also maintain protection against NK cell activity. This is due to the fact that HIV protein Nef downregulates human leukocyte antigen, HLA-A and HLA-B which present peptides that the majority of CTLs can recognize. However it allows the NK inhibitory MHC molecules (HLA-C, HLA-E alleles) to be presented on the surface of HIV-infected cells and thus block NK response\textsuperscript{19,20}. In contrary to NK and T cell synergy against viral infection, there are recent studies suggesting that NK cells play an inhibitory role on adaptive immunity. Research in NK depleted mice show enhancement on antigen presentation and improvement of T memory cell formation. In addition, IL-10 production of NK cells has been shown to induce T cell exhaustion. Therefore, a negative regulatory role for NK cells during both an acute and a chronic virus infection has been suggested\textsuperscript{21,22}.
1.1.3 The immune synapse

The recognition process of malignancies during surveillance in the body is obtained through a tight intercellular junction between a potential target cell and an NK cell that is called immune synapse (IS). Initially IS was described in 90s as the junction between T helper lymphocytes and antigen presenting cells (APC). T cell receptors interact with major histocompatibility complex molecules on APC that carry foreign or malignant peptides\(^{23,24}\). Later, formation of IS was observed also for other types of immune cells, i.e. NK and B cells\(^{24,25}\).

Extended studies of the synapse have revealed numerous functions and important intra- and intercellular communication processes during the assembly, formation and disassembly of IS. Ligand recognition, signal amplification, cytotoxicity activation or inhibition\(^{26}\), formation of membrane nanotubes\(^{27}\), receptor transfer\(^{28}\), cell-surface protein-lipid trafficking are some of these functions\(^{29}\). When an immune synapse between an NK cell and another cell is being formed, both activating and inhibitory receptors can be engaged simultaneously with ligands on the surface of potential target cell. If the ligated inhibitory receptors dominate the balance favors inhibitory signaling, and the cell seizes its activity, eventually detaches from the target cell, and the cytotoxic granules remain distributed all over the NK cytoplasm. In contrast, when the activating signaling dominates, the effector cell rapidly (within minutes) polarizes the cytotoxic granules towards the synapse and finally releases the lytic molecules that ensure target cell death\(^{26,30,31}\).

1.1.4 NK Killing pathways

Granule mediated death

One killing pathway is characterized by secretion of the content of lytic granules in the synaptic cleft between effector and target cell. These toxic granules initially existing in the cytoplasm of NK are complex organelles that contain a mixture of proteins (perforin, granzyme). They cooperate and together induce target cell ‘suicide’ by activating mechanisms of programmed cell death (apoptosis)\(^{9,32,33}\). Perforin has been characterized as a membrane disrupting protein that is released via exocytosis on the target cell’s membrane causing pore formation.

However the exact role of perforin in cell death has been much debated since it was purified in 1985. The initial concept was that perforin induces cell death by its membrane disrupting properties, due to osmotic instability caused by excessive ion uptake through the damaged cell membrane\(^{34}\). After the pro-apoptotic properties of granzymes were discovered, it has been widely accepted that the mechanism of granule-mediated apoptosis is performed by the synergy of perforin and granzyme proteins\(^{35}\). According to the initial synergy concept, granzymes are released, through perforin induced pores into the target cell’s
Immune surveillance

cytoplasm. Then they activate apoptotic cysteine proteases (caspases) that follow a path of biochemical processes that finally leads to cell death. It has also been suggested that the role of perforin in low (not lytic) concentrations is to permeabilize the target cell membrane so that granzyme can be endocytosed in the cytosol in the form of large vesicles.

Through the perforin induced pores there is flux of Ca\(^{2+}\) that triggers membrane repair response that can save the cell from necrosis and finally the release of granzyme in the cytosol induces apoptosis by caspase activation. The importance of apoptosis versus necrosis is that apoptotic cells cause much less inflammatory response compared to necrotic cells. Phagocytes (dendritic cells and macrophages) have specific receptors that are able to recognize changes on the surface membrane of apoptotic cells and remove them rapidly without causing inflammation. This function of cytotoxicity can be accomplished either through NK cell activation receptors binding or by recognition of IgG-opsonized cells through CD16 to enable antibody-dependent cell-mediated cytotoxicity (ADCC). Through ADCC, NK cell induced death is associated to adaptive immunity.

Receptor mediated death

The second killing pathway is induced by receptor-ligand signaling and is independent of lytic granule mediated death. FAS, (or APO-1 or CD95), is a surface membrane receptor that belongs to the tumor necrosis factor receptor (TNF-R) family and its role is to trigger apoptosis (by activating caspase cascade) when is ligated by its physiological ligand FASL. Additional members of this family include the death receptors DR4 (R1) and DR5 (R2) transducing apoptotic signals upon binding their soluble ligand, the TNF-related apoptosis-inducing ligand (TRAIL).

The exact mechanism of this process has been investigated and studies have shown that the ligation of FAS rapidly forms a ‘death-inducing signaling complex’ (DISC) which contains the apoptotic enzyme cysteine protease, caspase-8. Caspase-8 changes conformation, becomes fully activated and then undergoes autoproteolysis. This allows the enzyme to cleave, leave the DISC and continue its biochemical path in different compartments of the cell by activating other caspases finally leading to cell death.

Recent findings about the killing mechanisms of CTLs and NK cells have shown that the Fas pathway is always dependent on caspase cascade while granule mediated cell death can be independent of caspases induced apoptosis, through a non-nuclear pathway. In granule mediated cytotoxicity, granzymes A and B have been distinguished by their role in the apoptotic pathway. Granzyme A is considered to be responsible for caspase-independent death while granzyme B causes caspase-dependent apoptosis. Consequently if caspases are
inactivated either due to inhibitory viral proteins or due to mutation, the lytic granule pathway can insure rapid and efficient target cell death\(^2\).

### 1.1.5 Heterogeneity of NK cells

Today, human NK cells are defined as CD3\(^-\)CD56\(^+\) lymphocytes and they comprise nearly 10-15% of circulating lymphocytes but they also exist in tissues and organs. Upon activation from cytokines, NK cells can infiltrate from the blood stream in tumor containing tissues or viral infected tissues\(^46\). NK cells are divided in to two main subsets according to their functions of cytotoxicity and cytokine production. The intensity of expression of the neural adhesion molecule CD56 on NK cells, distinguishes them in either effective killers or immune regulatory cells. CD56\(^{\text{dim}}\) subset comprises around 95% of the total peripheral blood NK cells and expresses high levels of perforin and the Fc-\(\gamma\) lysis receptor CD16, (but low levels of cytokines), while subset CD56\(^{\text{bright}}\) expresses low levels of perforin and CD16 but is responsible mainly for cytokine production\(^30,47\). Besides the two main subsets, there are several more NK cell subpopulations characterized with different receptor repertoires and effector behavior\(^48\).

### 1.2 Microtechnology for biological research

A tremendous progress in techniques such as microtechnology, microscopy and computation technology has led to a breakthrough in biological studies over the last decades. Microfabricated devices are also known as micro-electro-mechanical-systems (MEMS), lab-on-a-chip microsystems or micro total analysis systems (\(\mu\)TAS). Microfabrication techniques were initially designed and developed for miniaturized integrated circuits in the field of microelectronics. However, to date they have been adopted and modified in order to develop tools with micrometer-scale resolution for biological research. Cell culturing inside microfabricated devices can mimic better the microenvironment existing in vivo systems, but also we can better control, manipulate and analyze the functions of the cells in different conditions according to experimental needs and biological questions. In addition, fluorescence (or phase contrast) imaging of cells in petri dishes or 96-well plates, limits the possibilities of visualization due to excess fluid and thickness of the plastic. Microsystems are currently being widely used for cell culture and manipulation, for genetic analysis, diagnostics and drug discovery through high-throughput drug screening\(^49-51\) etc. In addition, they play an important role as methods to investigate cells at the single cell level\(^52\).

### 1.2.1 Considerations in biological applications

Although many advantages exist in microtechnology for biological applications, there are some important considerations that should be taken in account...
during the design and fabrication of a device. As mentioned previously, it is crucial to develop in vitro culture conditions that mimic as closely as possible in vivo microenvironments. Several characteristics of in vivo conditions are not easy to be replicated. Continues cell-cell communication, nutrient supply, waste disposal and temperature-gas homeostasis are the main factors that ensure the physiological functions of cells. In microscale, the main transport mechanism is diffusion, where molecules move from regions with higher concentrations to lower concentrations. In contrast, in macroscale cell cultures, due to low SAV there is always a large volume of nutrients and gas supplies for the cells and waste products are easily diluted in the bulk solution.

Furthermore, due to high SAV in microcultures, the material interfaces between cells and the surrounding walls play an important role in the behavior of the cells. For example, proteins are seen to adhere on hydrophobic surfaces of microstructures and eventually denature and disengage from the walls. This continues phenomenon results the reduction of protein concentrations of the medium which can affect the culture conditions. Additionally, biocompatibility and cytotoxicity of microsystems are very important issues for biological applications. Residues from preparations processes (cleaning, sterilization) are tolerable in macrocultures, but can be toxic for cells in microenvironments due to low SAV\textsuperscript{53}. Another phenomenon that should be considered in microcultures, is evaporation of the medium due to small volumes and high temperatures. Evaporation can change the concentration (osmolarity) and PH of the media and cause cell damage\textsuperscript{54}.

However, although many cell types seem to be compatible with most microdevices, it is shown that the proliferation kinetics is not always in the same range as in macrocultures. Studies have shown that proliferation rates of cultured cells in macroscales differ from rates inside microdevices. An example is murine embryos that are shown to proliferate faster inside microchannels (in a rate that is similar to in vivo proliferation), than in traditional culture conditions. The opposite phenomenon has been observed in insect cells (Sf9) that are seen to proliferate faster in macroculture systems than in microchannels (in absence of flow)\textsuperscript{54,55}.

1.2.2 Single cell investigation

Due to a heterogeneity within cell populations it is very important to observe high numbers of individual cells in order to understand cellular processes and kinetics. Single cell analysis methods are emerging due to the insufficiency of averaged information of bulk cell solutions to describe these individual cell processes. For example, some cells have the ability upon stimulation to display unique repetitions of increase and decrease of Ca\textsuperscript{2+} concentrations over time. It is believed that these variations of Ca\textsuperscript{2+} concentrations can give valid information about the cells and their functions. However, this phenomenon
cannot be observed in bulk solutions due to differences in timing and response of the individual cells\textsuperscript{56}.

Multiwell microstructures used for cell cultures, give the possibility to follow large numbers of cells in time scale of several days and collect valid statistical information. It is important that the information about the cells can be obtained under the same culture and preparation conditions (temperature, staining, seeding), in order to ensure the reliability of the observations\textsuperscript{57}. Furthermore, the benefits of using miniaturized systems include the consumption of smaller volumes of sample, growth factors, reagents, and have smaller space requirements. In addition, reactions can be more effective due to the large surface-to-volume ratio (SAV)\textsuperscript{58}.

1.2.3 Materials and fabrication

Several techniques have been developed using different materials and processes for microfabrication. When a device is built from a bulk material (substrate) is called bulk micromatching, instead when the fabrication process is held entirely on the surface of the substrate, the method is called surface micromachining.

However, a wide range of devices is built with a combination of both fabrication techniques and different materials such as glass, silicon, metals and polymers. The main processes of fabrication consist of photolithography, etching and bonding.

\textit{Photolithography}

In photolithography, the idea is to transfer a designed pattern on the material of use. The first step includes the copy of the pattern onto a mask, which is for example a glass plate or printed plastic, with a photodefinable opaque surface that is modified and formulates the designed pattern. The next step includes the spin-coating of a substrate with a photoresist material (photosensitive organic polymer), which after being exposed to UV light through the mask, it replicates the pattern on the surface of the substrate. The resulting substrate pattern can be used as a protective layer for etching processes or as a stamp master for transferring the pattern onto soft materials by peeled-off methods (soft lithography).

\textit{Etching}

Etching is the process of the creation of a pattern on a substrate; either by using liquid chemicals (wet etching) or gas-phase chemicals (dry etching) which is the method used for the multiwell chips used in this Thesis. Either method can lead to isotropic or anisotropic etching. Isotropic corresponds to the method where etching is equally performed in depth as well as at the sides of the substrate; in contrast, anisotropic etching proceeds towards a desired direction.
Usually wet is more selective than dry etching, however for anisotropic, dry etching is considered to be more appropriate method.

**Bonding**

An important process in microfabrication technology is the bonding of two or more substrates to form a hermetically sealed system. Several materials can be bonded together, (usually glass and silicon) either by using intermediate layers or by treating the primary materials with high temperature, pressure or with high electric field. In these cases due to high temperatures, the materials of use should have similar thermal expansion coefficients. Several other bonding methods exist depending on the nature of the materials and the purpose of the device. PDMS can be reversibly hermetically bonded to glass or two PDMS membranes can be irreversibly hermetically bonded to each other after being oxidized and brought in contact\(^{49,52,59}\).

### 1.2.4 Ultrasonic manipulation

The work presented in this Thesis utilizes ultrasonic manipulation for the positioning and aggregation of cells. Ultrasonic manipulation of suspended particles is based on the time-averaged acoustic radiation force. This force originates from a non-linear effect in the acoustic pressure field and was first described by Lord Rayleigh\(^60\) in 1905. Later, a very useful theoretical model was presented by Gor’kov\(^61\) in 1962. This model is described in more detail in Paper V. In brief, Gor’kov’s theory describes the primary acoustic radiation force acting on a small particle, dependent on the sound field and material properties of the particle and the surrounding suspension medium. This model is valid for arbitrary sound fields and a single, spherical particle with known material properties. When the primary radiation force is applied on particles in suspension, it drives them in a direction parallel with the gradient of the acoustic pressure amplitude, and has a direction and magnitude defined by the acoustic contrast factors. Since a gradient is preferred, standing-waves are most often used. The acoustic contrast factors depend on the ratios of the compressibilities and densities between the particle and the fluid, respectively. Furthermore, the magnitude of the primary radiation force also depends on the volume of the particle as well as the ultrasound frequency.

An important phenomenon in standing-wave devices is resonance. In a resonator, waves of the same phase and wavelength arithmetically enhance the displacement amplitudes when they interfere. This is a way of signal amplification. In the case of microfluidic microdevices a wave propagation signal produced by a moderate energy performance transducer would not have the requirements to cause a particle trapping effect. Since the displacement amplitude of an acoustic wave within a channel can be highly amplified through the acoustic resonance of standing-waves, they are widely used for particle
manipulation in microfluidic systems. The requirements to produce high acoustic radiation forces within a channel, besides the wavelength-width matching for generating resonance, the acoustic impedance also plays a major role. The acoustic impedance can be simply described as the ability of a material to reflect an incident wave\textsuperscript{62}. Thus, interfaces with mismatched acoustic impedances can reflect better and produce a better signal amplification within the channel.

In a standing-wave field, the primary radiation force drives most suspended particles either to the pressure nodes or the pressure anti-nodes, depending on the contrast factors. In principle, particles denser than the suspension medium are driven to the pressure nodes, while particles less dense than the suspension medium are driven to the pressure antinodes. However, in simple standing-wave fields (such as a one-dimensional field), the pressure nodes and the velocity antinodes are co-located. In one-dimensional (1D) standing-wave fields, the clusters typically take the form of flat monolayers in the pressure nodal planes. This is presented in Figure 1 where acoustic resonance occurs within a microfluidic channel of a length corresponding to half a wavelength of the actuated frequency (Figure 1A). Therefore, a single pressure node is formed. Primary acoustic radiation forces direct the particles towards the pressure node and distribute them along the nodal plane (Figure 1B). A secondary radiation force that occurs (Figure 1C) is generated by particle-particle interaction and leads particles to attract each other and form a more compact monolayer in the nodal plane (Figure 1D)\textsuperscript{63}. This force is much weaker than the primary radiation force and it is generated only in short distances between particles. However, the secondary force contributes to the stabilization of monolayer aggregates in 1D resonators.

The designs used in this Thesis utilize 2D resonances for ultrasonic manipulation of cells. In 2D or 3D standing-wave fields, the cluster shapes are more complicated to predict or control\textsuperscript{64}. Furthermore, the theoretical model\textsuperscript{61} is valid for spherical particles with well-known material properties (density and compressibility) suspended in an inviscid fluid. But cells have unknown material properties, or if known, their material properties have a wider distribution than for synthetic particles (e.g., polystyrene). In addition, the material properties of cells are also dependent on many external and internal factors. Therefore, it is difficult to predict the contrast factors for cells. Experiments that have been performed in order to estimate the acoustic radiation forces on cells, have shown that in a given acoustic field the acoustic radiation force is roughly a few times smaller for cells than for polystyrene particles of similar size, and that the corresponding trapping time is expected to be a few times longer. A similar approach has recently concluded that the radiation force was 1.5 times smaller for red blood cells, and between 2 and 4 times smaller for different types of cancer cells relative to the force on equally
sized polystyrene\textsuperscript{65}. However, these studies are still preliminary, and more accurately measured acoustic contrast factors for cells are still to be determined.

![Figure 1. Ultrasonic manipulation of particles in a 1D half-wave resonator. (A) An illustration of cross-section of a microchannel filled with green particles. (B) The microchannel is actuated with a frequency matching the channel width i.e., the width of the channel corresponds to half a wavelength ($\lambda/2$). (C) A standing wave is formed and the primary radiation force (red arrows) is directing the particles towards the pressure nodal plane in the center of the channel and oriented vertically. (D) The secondary radiation force (blue arrows) produced by particle-particle attraction due to wave scattering on particles, directs them in the center of the nodal plane forming a monolayer.](image)

**Applications**

The ultrasonic manipulation of particles or cells is usually referred as acoustophoresis\textsuperscript{66}. Particle or cell manipulation systems based on acoustic standing-waves, are used today in various applications such as separation, focusing and trapping\textsuperscript{62,67-70}. One of the most common application of acoustic manipulation in microfluidic chips is particle separation, which is feasible because of the different particle characteristics (i.e., density, compressibility and volume). Due to the large diversity among the blood components, blood plays an important role in homeostatic regulation while providing a good source for markers indicating the overall health condition. Therefore, separation techniques can be useful if applied in blood samples for clinical diagnostics\textsuperscript{50}. Examples include separation of platelets from peripheral blood progenitor cells\textsuperscript{71}, apheresis of blood components to collect pure plasma for antigen detection\textsuperscript{72}, and detection of circulating tumor cells for cancer diagnostics\textsuperscript{73}. A great challenge in the medical research field is to employ new research tools for detection of low concentrations of bacteria in blood for early sepsis diagnosis. Bacteria detection in acoustophoretic systems is challenging due to the small bacteria size. However, there are ways to overcome the problem by exploiting the systems potential to secondary effects, such as
streaming and secondary radiation forces\textsuperscript{62}. Another reported application involves the generation of HepG2 cell aggregates in an ultrasonic trap for short time periods (5 minutes), followed by encapsulation in hydrogels in order for the aggregates to develop into solid tumor for subsequent use in toxicology studies\textsuperscript{74,75}.

1.3 3D Biological culture systems

Current bio-assays used for research on cellular behavior within an organism, are based on the assumption that cell monolayers behave in an analogous way with the three-dimensional structure of real tissues \textit{in vivo}. The transition from two-dimensional (2D) to three-dimensional (3D) cell culture systems is motivated by the need to imitate the biochemical and mechanical structure and microenvironment of living tissues that is lacking from current 2D culture systems. Cells within a tissue interact with other cells and the extracellular matrix (ECM) through a 3D communication network that is crucial for the tissue survival and development\textsuperscript{76}. 3D cultures gain territory in biological studies associated with cell migration and adhesion. Additionally, in tumor cell biology, in order to better understand the microenvironment of solid tumors \textit{in vitro}, researchers need to mimic the 3D structure of the developing tumor. 2D cultures that are commonly used are inadequate to recreate this microenvironment and thus the outcomes of the experiments can be insufficient or misleading. Furthermore, solid tumor spheroids show increased drug resistance comparing to 2D structures due to tight cell-cell contacts and interactions\textsuperscript{77,78}. In addition, cell adhesion and migration mechanisms can be better elucidated through a multicellular 3D structure since cell transmembrane adhesion proteins are shown to be distributed in a diverse manner than in cell monolayers\textsuperscript{77}.

Cell populations orchestrate behaviors such as migration, proliferation and apoptosis in a way that is crucial for developing a multicellular tissue. Specifically for epithelial cells, 3D culture systems are essential in order to elucidate how cellular functions are regulated during epithelial morphogenesis\textsuperscript{79}. Moreover, comparison among gene expression of cells cultured in 2D and 3D systems indicate different expression levels. For example, in the case of melanoma cultures in spheroids, results indicate that the upregulated genes found in spheroids correlate to upregulated genes in real tumors \textsuperscript{80}, while mammary epithelial cells in 3D cultures induce expression levels of mRNA analogous to those in breast tissue\textsuperscript{81}.

Previous models in developmental biology suggest that the growth and morphology of an embryonic tissue is ‘pre-programmed’, however the last decade the models have been revised by considering the effects of interaction and communication between cells and the extracellular microenvironment\textsuperscript{82}. In addition to the effect of microenvironment on tissue morphogenesis, experiments indicate that the same phenomenon exists in cancer biology where melanoma cells responded to an embryonic microenvironment and reversed to
healthy phenotype\textsuperscript{83}. Another challenge for cellular systems rises in pharmaceutical companies in order to increase the success rate of drug development in early stage. Cell based assays are expected to improve drug screening processes by direct cell-specific response\textsuperscript{84}.

**Available methods for 3D cultures**

Due to the large demand, several techniques for 3D cultures have developed and optimized over the last decades, thus today there are many commercially available tools for multicellular 3D cultures for applications in biology and medicine\textsuperscript{85}. Today there are many commercially available tools for 3D cultures for applications in biology and medicine\textsuperscript{85}. Most of these methods require physical scaffolds around the cells such as proteins and hydrogels in order to grow in a 3D structure. However, such scaffold-based systems have limited imaging possibilities and usually require further chemical treatment to remove the scaffold. Besides scaffolds, other 3D culture methods require cell adhesion resistant surface coatings such as agarose and poly-HEMA\textsuperscript{86}. For example, coatings may be applied in microstructure patterns or combined with conical-bottom plates\textsuperscript{87}.

Other methods are based on cell growth into 3D structures by avoiding cell-substrate contacts using external forces. One example is the ‘rotating vessel’ method where cells are placed in a rotating cylinder filled with medium and extracellular matrix-coated beads as scaffolds. Since sedimentation is not feasible due to the rotating fluid, cells and coated beads tend to accumulate into clusters and consequently develop into 3D cultures\textsuperscript{88}. Another commonly used method is the “hanging drop”, where cells without scaffolding, assemble into spheroids by gravity in drops within an upside-down oriented microplates\textsuperscript{89}. This simple system, although it does not require surface modification or scaffolds, is limited by the difficulty to keep the drops stable over extended culture times, in particular to prevent evaporation. Furthermore, like most scaffold-based methods, the ‘hanging drop’ is not directly compatible with live cell imaging. Another reported method uses magnetic levitation of a hydrogel composed of cells, magnetic iron oxide, gold nanoparticles, and bacteriophage\textsuperscript{90}. This method is promising but currently not scalable into high-throughput parallel 3D cell culture.

**1.4 Fluorescence imaging**

Fluorescence is the optical phenomenon of light emission by a molecule after being illuminated. Fluorescence occurs when a photon (basic unit of light) that carries a sufficient amount of energy, excites the electrons of a molecule with fluorescent properties into a higher energy state. The excess energy is released in the form of another photon and the electrons returns to the initial state (ground state). During the process, the electron loses energy in the form of heat and vibration, thus the emitted photon carries lower energy than the absorbed photon.
The term fluorescence was coined, after the mineral fluorspar (calcium fluoride CaF₂), by the British mathematician and physicist George Stokes in 19th century. Stokes observed that a solution of quinine sulphate although it is perfectly transparent, emits a blue-color light when illuminated by ultraviolet wavelength. According to Planck’s law the energy is reciprocal to wavelength. Therefore, the emitted photon since it carries lower energy than the excitatory photon, emits light with longer wavelength. The wavelength difference between the absorbed and the emitted light is known as Stokes shift. Fluorescence-based imaging techniques have been rapidly developed and today are being widely used for biological observations. The phenomenon of Stokes shift makes it possible to efficiently separate the excitation light from the emission light and therefore fluorescence is highly selective above other imaging techniques.

Fluorescence microscopy initially allowed scientists to investigate substances such as minerals, crystals, vitamins and other inorganic compounds that have fluorescent properties (auto-fluorescent). To date, tissue components that do not have fluorescent properties can be treated with fluorescent molecules in order to be detected and observed by fluorescence microscopy. For example, a wide range of fluorescent molecules allows labeling and multi-color visualization of different parts of the cell such as the plasma membrane, nucleus or cell organelles. Genetic modifications on cells allow them to express fluorescent markers, such as green fluorescent protein (GFP), that are bound to any cell protein of interest. Protein expression, distribution and localization can also be studied by immunofluorescence, where antibodies that are tagged with fluorescent molecules are used to detect protein kinetics.

For live cell imaging it is crucial to consider and maintain the cells’ health in order to provide valid information that reflects closer to in vivo conditions and real functions of cells. Cells are sensitive to photo-damage especially in the presence of fluorophores, however there are several ways to limit the problems, for example by reducing the duration and intensity of the illumination. In addition, excitation of the specimen with visible light has been shown to be less harmful for living cells than being exposed to ultraviolet light. In fluorescence microscopes the sample is directly excited by a light source and both transmitted and emitted light are collected by the objective.

To date most fluorescence microscopes are epifluorescent. Epifluorescence is an optical set-up for a fluorescence microscope where the excitatory light passes from above (or from below in inverted microscopes) through the objective and then on the specimen. The fluorescent light generated in the specimen, is collected by the same objective that is used for the excitation of the sample. A combination of wavelength filters between the objective and the detector ensures collection of the emitted light and rejection of the excitation light. The main filtering compartments of a fluorescence microscope are the excitation filter, the dichroic mirror (or dichromatic beam-splitter) and the...
emission filter. Laser scanning confocal microscopy (LSCM) is a more developed form of fluorescence microscopy that offers several advantages, such as control of depth of field, elimination or reduction of scattered and out-of-focus light and thus gives the ability of collection of stacks of images from thick specimen and three-dimensional reconstructions. In confocal microscopes a laser beam penetrates the specimen and focuses onto a point on the desired plane. Since the laser beam travels through the specimen in order to reach to the depth of interest, it illuminates other sections of the sample. In order to avoid detection of the emitted light originated from points close to the focal plane, an adjustable detector aperture (pinhole) is placed before the detector. The pinhole ensures the rejection of the out-of-focus light and reflection glare, but allows the light emitted by the excited point on the focus plane to be detected.\textsuperscript{95}
Chapter 2: Materials and Methods

2.1 Micro-well microplate designs

Since populations of cells carry a heterogeneity that cannot be resolved in tests performed in bulk solutions, a more individual approach to investigate this heterogeneity is needed. The aim of using microwells is to isolate high numbers of individual conjugates of NK-target cells and observe their behavior and interactions in a time scale of several days. The chips were fabricated with silicon and glass with the use of photolithography and etching. Depending on the application and the questions addressed, different designs of the multiwell microplates have been fabricated and used. For screening and recording high numbers of cell-cell interaction individual events, the smaller size of well design was used. The first generation of chips were composed of four compartments with two different well dimensions and wall thicknesses. An illustration of the chip and the holder is shown in Paper II in Figure 3.

The dimensions of the chip were $24 \times 24$ mm$^2$ surface area and $470 \ \mu$m thickness in total (silicon $300 \ \mu$m and glass $170 \ \mu$m). The chips were separated in four different compartments of $10 \times 10$ mm$^2$ surface area and contained large numbers of squared-shaped wells. Two chip compartments were comprised of $80 \times 80 \ \mu$m$^2$ wells where the distances between the wells (walls) were $40 \ \mu$m in one compartment and $80 \ \mu$m in the other. The other two compartments of the chip was comprised of $50 \times 50 \ \mu$m$^2$ wells and the distances between the wells are again $40 \ \mu$m and $80 \ \mu$m. The upper openings of the wells were somewhat wider than the bottom of the wells due to the process of etching. The dimensions, the distances of the wells and the total well number in each compartment are shown in table 1. A custom fabricated aluminum holder ensured easy handling and securing the chip under the microscope. More than that, the holder was designed to provide a reservoir of medium above the wells to provide easy exchange of nutrients during long cultures.
Micro-well microplate designs

Table 1. Dimensions and characteristics of the first generation of silicon-glass chips.

<table>
<thead>
<tr>
<th>Chip compartments</th>
<th>Well dimension ($\mu m^2$)</th>
<th>Well distance (wall thickness)</th>
<th>Total number of wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80 x 80</td>
<td>40</td>
<td>6889</td>
</tr>
<tr>
<td>2</td>
<td>80 x 80</td>
<td>80</td>
<td>3969</td>
</tr>
<tr>
<td>3</td>
<td>50 x 50</td>
<td>40</td>
<td>12321</td>
</tr>
<tr>
<td>4</td>
<td>50 x 50</td>
<td>80</td>
<td>5929</td>
</tr>
</tbody>
</table>

The multiwell microchip can be very suitable for screening high numbers of individual events, however it is limited for long cultures and synchronization of the events. Although the small well geometries would ensure cell interaction at some point, the exact time of interaction could not be determined or scheduled. Furthermore, due to the small size, increased numbers of cells after long culture periods can overfill the wells.

An updated design of the multiwell chip was fabricated to be used in an ultrasonic cell manipulation device. The ultrasonic manipulation technique was previously used for trapping particles or cells in flow through channels\cite{96,97}. The current method involves acoustic radiation forces that direct cells to the pressure nodes of acoustic waves in resonance, within each of the wells. The purpose of implementing the multiwell in the ultrasonic device was to avoid limitations of the small wells related to migration steps and timing of cell-cell interactions. Directing cells in the center of each well by acoustic radiation forces could overcome the migration step and assist cell conjugation, simultaneously in all wells.

The multiwell microplates used in the ultrasonic-actuated cell manipulation device were made of silicon and glass, with equal silicon wafer thickness of 300 $\mu m$ as the first generation of chips and a glass thickness of 175 $\mu m$. In order to obtain acoustic standing-waves in each of the wells, the ultrasonic frequency applied on the device should correspond to the well dimensions as explained in detail in §1.2.4. The dimensions of the multiwell microplate was $22 \times 22$ mm$^2$ and contained 10x10 centrally positioned square wells covering an area of $3.9 \times 3.9$ mm$^2$ (each well was $300 \times 300$ $\mu m^2$ bottom area with $10 \mu m$ corner radii and separated by $100 \mu m$ walls). This design was operated with approx. 2.5 MHz ultrasonic central frequency. Two other designs of silicon-glass microchips were used with slightly different well size and shape. The well dimensions were $350 \times 350$ $\mu m^2$ and the well shapes were either square with $10 \mu m$ corner radii or slightly rounded concave wells. The last chip designs were operated with approx. 2.2 MHz central frequency.
2.2 Preparation of microchip for cell experiments

The cleaning process of the chips varied depending on the well size. As expected, smaller wells (50, 80 μm) require more steps and longer times to remove dead cell debris than the larger well chips (300, 350 μm). A common cleaning process for the different wells is described below.

Unused or cleaned and dried chips were placed in 6 mm diameter petri dishes with ethanol solution of 70%. A submerged chip was then placed in an excicator coupled to vacuum pump so that the air bubbles would be removed from the bottom of wells. When the air bubbles were removed, the chip was placed in a beaker with approx. 80 ml sterile water and then in the ultrasound device for 10-15 minutes. Afterwards the chip was transferred in a petri dish with cell culture medium. The chip was kept in the medium for more than 1 hour before loading and the medium was exchanged several times to ensure complete removal of water molecules that could harm the cells. In the case of the multiwells chips used for ultrasonic manipulation the medium was added in the basin above the wells defined by the PDMS frame (see Figure 2 and Figure 3).

The used small multiwell chips were rinsed with water and then put in a sodium hydroxide solution (5 mol/l) for 10-20 minutes (NAOH was not necessary for cleaning the 300 μm wells). Afterwards the chips were washed thoroughly with sterile water and were placed in 200 ml beakers with 70% ethanol solution.

For further cleaning, the beaker was placed in an ultrasonic device for 1 hour at 40-50°C. The ethanol was then replaced with sterile water and the beaker was placed in the ultrasonic device for another at least 10-15 minutes in order to remove all the residues of ethanol from the wells. During the procedure of the ultrasonic cleaning, air bubbles were formed in most of the wells. As a final step, each chip was placed in a petri dish with cleaning medium and then in a vacuum chamber until the air bubbles were completely removed from the bottom of wells (approx. 15-30 minutes). When the chip was prepared one or more days earlier than the scheduled experiment, the air bubbles were noticed to spontaneously disappear from the wells, without using the vacuum pump. For the larger well chips, the bubble could be removed even with resuspension of the water or medium using a 100 μl pipet.
2.3 Cell preparations

2.3.1 Cell cultures

*Human primary NK cells*

The aim of this study was to investigate the functional properties of natural killer cells such as NK-mediated cytotoxicity against individual cancer cells as well as NK cell infiltration and killing of solid tumors. Since polyclonal NK cells harvested from peripheral blood maintain a heterogeneity within their population comparing to cell lines, primary human NK cells were used rather than NK cell lines. The YTS NK cell line that was transfected to express the KIR2DL1 inhibitory receptor was used only for studying the inhibitory immune synapse formed with 721.221/Cw6-GFP as explained below. Human primary NK cells were isolated from fresh, lymphocyte enriched buffy coat residues derived from healthy donors.

As a first step, peripheral blood mononuclear cells (PBMC) were isolated from red blood cells through centrifugation on Ficoll-Paque Plus gradient (GE Healthcare) for 20 minutes at 720 × g with acceleration and deceleration set to zero (GE Healthcare). PBMC were collected in 50 ml falcon tubes, they were resuspended in PBS and centrifuged for 10 min in 400 × g. The washing step was repeated 3 times to remove platelets from the supernatant. The final selection of NK cells from PBMCs was performed by negative immunomagnetic bead isolation (EasySep Human NK Cell Enrichment Kit, StemCell Technologies). Briefly, a cocktail of antibodies was added to the solution that could recognize and bind to all PBMCs but not to NK cells. PBMCs were before counted in a burker chamber and the right volume of the antibody cocktail was added to ensure binding to all existing PBMCs thus obtain high NK purity. Then a solution of magnetic beads designed to attach to the antibodies was added to the PBMC-antibody mixture. Final step was the positioning of solution-containing tubes in a strong magnetic field trapping the magnetic beads coupled to the unwanted PBMCs and therefore only pure and unmodified NK cells were collected.

Previously, after the initial step of PBMCs separation from whole blood, besides PBMCs the blood plasma was also collected and heat-inactivated in 56 °C for 30 min. The serum was then centrifuged and filtered to remove any dead cell residues and inactivated proteins in order to be used as component of NK growth medium. The NK cell culture medium contained Iscove’s modified Dulbecco’s medium (IMDM, I3390, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% of the treated, autologous human serum, 2 mM L-glutamine (G7513 Sigma-Aldrich, St. Louis, MO, USA), 100 U ml⁻¹ penicillin–100 mg ml⁻¹ streptomycin (P4333; Sigma Aldrich, St. Louis MO, USA), 1×non-essential amino acids (M7145, Sigma-Aldrich, St. Louis, MO, USA) and 1 mM sodium pyruvate (S8636, Sigma-Aldrich, St. Louis, MO, USA).
Since the NK cells must be activated in order to grow and proliferate \(^{98}\), in addition to the above components, 200 U ml\(^{-1}\) of the human cytokine Interleukin-2 (IL-2) (PeproTech, Rocky Hill, NJ, USA) was added to the final NK culture medium. NK cells were tested for purity and cytotoxic capacity seven days after isolation. Purity was confirmed with fluorescence-activated cell sorting (FACS) to be 99.5% CD3-CD56\(^+\) and their killing capacity was also verified by conventional cytotoxicity assay against 221 cells\(^{99}\).

**Cell lines**

Cell lines were used for a variety of applications over the entire project including microdevice viability studies, ultrasonic trapping efficiency tests, as models for the formation of 3D multicellular solid tumors and as target models for analyzing NK cell functions against single cancer cells and solid tumors.

The human B lymphoblastoid cell line 721.221 (referred to as 221) was used for testing the trapping efficiency of the ultrasonic device for non-adherent cells in different actuation voltages and as targets for testing NK cell cytotoxicity in Paper IV. The 221 cell line is deficient in endogenous expression of MHC class I proteins\(^{100}\) and since it is susceptible to NK-mediated killing they are commonly used as targets to study NK cell cytotoxicity \(^{101,102}\). However, 221 cells when are transfected to express the MHC class I molecules HLA-Cw6, they can be ligated with KIR2DL1 inhibitory receptor on NK cells and induce formation of an inhibitory synapse \(^{103}\). Therefore, for imaging the inhibitory immune synapse we have used the NK cell line YTS\(^{104}\) transfected to express KIR2DL1. As conjugation target we used 721.221 cells genetically modified to express MHC class I molecules tagged with green fluorescent protein (GFP)\(^{105}\).

The HEK293T is an adherent cell line, a variant of the HEK 293 lines, originally derived from human embryonic kidney cells \(^{106}\). The HEK293T cell line in addition contains the SV40 Large T-antigen\(^{107}\). HEK293T cells were used for viability studies and as models to investigate trapping efficiency of adherent cells in the ultrasound device.

The HepG2 cell line is derived from hepatocellular carcinoma tissue of a 15 year old Caucasian patient. Due to their characteristic properties to form solid multicellular structures, HepG2 cells have been used as models for ultrasound-assisted formation of solid tumors in the multiwell microplate. Furthermore, HepG2 cell in monolayer cultures have been used by other research groups as target cells for NK cell-mediated killing to investigate the NK killing mechanisms against hepatocellular carcinoma\(^{108}\). In our study, solid tumors composed of HepG2 cells have been used as targets to investigate NK cell infiltration and cytotoxicity properties against multicellular solid tumors.

The 221, HEK293T and YTS cell lines were cultured in RPMI-1640 (SH30027 Thermo Scientific, MA, USA) supplemented with 10% fetal bovine serum (SV30160; Thermo Scientific, MA, USA), and 100 U ml-1 penicillin-100 mg ml-1 streptomycin, 1× non-essential amino acids and 1 mM sodium pyruvate. HepG2 cell line was cultured with additionally added 2mM L-glutamine. All
cells (primary and cell lines) were maintained in suitable environmental conditions of 37°C and 5% CO₂.

2.3.2 Cell labeling

For resolving live from dead cells we used two different viability dyes, the calcein green-AM and the calcein red-orange-AM (Invitrogen, Carlsbad, CA, USA). Calcein-AM is a highly lipophilic dye that can rapidly enter viable cells and convert by intracellular esterases to produces an intense fluorescent signal. In addition, calcein can only be retained by cells with membrane integrity and it is rapidly ejected from damaged or dying cells. Due to these characteristic properties, calcein has been widely used in live cell imaging and the last decades has replaced conventional methods for evaluation of cell viability, cytotoxicity and for quantifying apoptosis.

In order for dead target cells to be fluorescently visible after calcein release, in addition to calcein, the long-term cell labeling dye far red DDAO-SE (Invitrogen, Carlsbad, CA, USA) was used. Therefore, NK cells were stained with red-orange-AM and target cells with both calcein green-AM and far red DDAO-SE. Interestingly, although far red DDAO-SE has a low intensity on alive cells, it slightly increases short after a cell has died. This is an extra benefit for evaluation of live/dead cells. Furthermore, we observed that far red DDAO-SE is greatly fluorescent on cells that are dead during the staining process, so these cells can be easily identified and excluded from the viability evaluation. An overview of the staining procedures for different experimental approaches is summarized below.

For cell labeling in solutions, cells were washed twice in RPMI-1640 and centrifuged for 10 min at 400×g to a pellet. The staining solution, prepared by dissolving the dyes in warm (37 °C) RPMI-1640, was added directly to the cell pellet. Dye concentrations used for monitoring of NK cell mediated killing were 0.25 μM for calcein green, 0.32 μM for calcein red-orange and 1 μM for DDAO. For the adherent and non-adherent cell trapping experiments 293T cells were stained with 0.5–1 μM calcein green. For the long-term tracking experiments of cell clusters, we used 0.5 μM for both calcein green (for 221) and calcein red-orange (for NK cells) and 10 μM for DDAO (for 221). For high-resolution imaging of the immune synapse, the YTS cells were stained with 6 μM DDAO. For all stainings of cells in solution, the procedure was the following: cells were resuspended and incubated for 10 minutes (except YTS cells, which were incubated for 15 minutes). Stained cells were washed twice in RPMI-1640 and resuspended in culture medium before being used.

A different staining procedure was followed for the solid tumors since they were stained directly in the multiwell chip. The growth medium was removed as gently as possible using a 100μl pipet. Then RPMI-1640 was added slowly on top of the wells and resuspended with the opening of the pipet tip facing the PDMS top to minimize turbulence. The procedure was repeated three times and finally the staining solution was added. The dye concentrations were 0.25 μM for calcein green and 1 μM for DDAO in 37 °C RPMI-1640. The solution was
resuspended, removed and added again. This process was repeated 3 times and finally the chip was placed in the incubator for 30 minutes. After that the chip was again washed two more times with RPMI and finally growth medium was added and the chip before sealing it with a cover glass.

### 2.3.3 Cell seeding in the chip

Depending on the desired number of cells in the wells, different volumes and cell concentrations were pipetted in the chip. Especially for the experiments that required single cells per well, the desired numbers were obtain in a more controlled way by inspection of the wells in an optical microscope while seeding. When the required number of cells was achieved, the excess cell solution was removed and replaced with growth medium. Cell sedimentation in the small wells was more complicated due to the small well openings. The 221 cells although they are non-adherent they show a tendency to form clusters hence blocking the opening of the wells. This issue was avoided by resuspending the cell solution until the desired number of targets in the wells was achieved (1-3). NK cells were easier to be seeded due to their smaller size and because they do not tend to form clusters even when cultured in high concentrations.

Again, for the small well chips another complication had to be resolved; due to the small surface area of each well, it was probable that some NK cells would encounter and form contacts with the target cell immediately after seeding, thus killing could occur before imaging. To avoid information loss, NK cells before seeding they were kept for 10-15 minutes at +4 °C in order to block their migration and killing activity until imaging was initiated.

### 2.4 Ultrasonic manipulation systems

Our manipulation system involves 2D standing-waves generated within each of the wells of the multiwell plate. Bellow, I will describe the different transducer designs, their components, and the operation settings of each device.

#### 2.4.1 Device designs

**Wedge-transducer**

The ultrasonic manipulation devices were comprised of two different transducer designs, the wedge and ring-shaped transducers. The wedge-transducer used in Vanherberghen et al., study (Paper I) is described in reference\textsuperscript{112}. A more robust wedge-transducer design was fabricated in Paper IV, where the aluminum wedge was replaced with a titanium wedge of 45° angle, 20 × 7 mm\textsuperscript{2} base area was glued with conductive, silver glue to a piezoelectric ceramic plate (length 15, width 5, and thickness 0.80 mm). The thickness corresponds to generation of 2.5 MHz sound waves. In addition, by
replacing the BNC type connector with the smaller BNC type and also the connections to the piezo from silver glue to soldering material, we increased the transducer efficiency. Then by using the epoxy glue we could secure the cables on the piezo element making the transducer more robust and reusable for several times. The chip was glued to the holder with a water-soluble conductive adhesive gel (‘Tensive’, Parker Laboratories, USA) with a thin cork frame between the chip and the holder to avoid chip damage and energy loss. The same glue was used to attach the wedge-transducer on the chip. A step forward for the new device design was the permanent bonding of the PDMS gasket surrounding the wells. By securing the gasket, leakage and detachment was completely avoided. This way the wells could be washed directly while attached to the device without removing the chip or transducer from the holder, allowing the device to be reused for several experiments. The chip design that was used in the wedge transducer device was the squared-shaped wells with $300 \times 300 $ $\mu m^2$ well openings. The wedge-transducer design and its components is explained in detail in Figure 2

Figure 2. Illustrations of the wedge-transducer device and its components. (A) The device set-up. The zoomed-in region shows a NK-target cell interaction event. (B) (1) Connecting cable. (2) Piezoelectric crystal. (3) Titanium wedge. (4) Cover glass to avoid medium evaporation. (5) PDMS frame plasma bonded around the wells. (6) The multiwell chip. (7) Cork frame in order to avoid energy loss. (8) The aluminum holder.

**Ring-transducer**

The ring-transducer device is an even more robust system since the piezoelectric element is permanently integrated in the aluminum holder and the connector cable is built-in the device. The characteristic ring-shaped piezoelectric element permits light to pass through the central opening through the wells allowing microscopy imaging. In addition, since it is free of water-soluble conductive adhesive gel, it can operate inside high humid environments like a cell incubator. The multiwell designs used in the ring-transducer device were the $350 \times 350 $ $\mu m^2$ with either squared-shaped wells or with slightly rounded concave wells. The ring-transducer device is presented in detail in Figure 3.
Figure 3. The ring-transducer device and its components. (A) Illustration of the ring-transducer set-up. (B) (1) Springs. (2) Aluminum clip. (3) Polymethyl methacrylate (PMMA) spacer. (4) Cover glass. (5) Micro coaxial (MCX) connector (6) PDMS frame. (7) 100-well microplate. (8) Ring-shaped PZT piezo crystal. (9) Aluminum baseplate.

2.4.2 Device operation

After cells were released in the PDMS basin above the wells, they were allowed to sediment but not adhere (in the case of adherent cells). Next, the transducers were actuated by a signal generator (DS345, Stanford, USA) and the settings of the generator were optimized for each transducer design, the well design and the cell application. As a result of the ultrasound actuation in the wells, a single-node was generated in each of the wells approximately in the center (Figure 4D, Figure 5).
Figure 4. A Schematic illustration (not to scale) of the different steps of the cell handling method. For simplicity, only three out of a hundred wells are shown (vertical cross-section view), and only the PDMS frame and the multiwell microplate. (A) The microplate is first primed with cell medium shown in purple. (B) The cell suspension is added as a small drop from a pipette tip. (C) Cells sediment by gravity down into the wells. The average number of cells per well is controlled by the cell concentration in the added drop in (B). (D) When ultrasound is turned on, cells are trapped, aggregated and centrally positioned in each well.

The wedge-transducer was actuated with a central frequency of approximately 2.55 MHz which corresponds to half a wavelength in the wells. The transducer was driven at up to 10 V_{pp} (volts peak-to-peak), either at selected single frequencies within the interval 2.55–2.65 MHz, or with linear frequency sweeps of 100 KHz around the central frequency at a rate of 1 kHz. Two thermocouple probes were attached on the silicon chip close to the PDMS gasket, one at the side of the wedge (between the wedge and the PDMS) and the second probe was placed on the other side of the PDMS away from the transducer. This way it was possible to follow the temperature gradient from the wedge-transducer through the wells. The probes were connected to a precision measuring instrument (P655-Log, Dostmann electronic, Germany) and the temperatures were presented real time during the entire cell experiments on a DE-graph (Rel. 1.3.3. software). Similar method was used for the ring-transducer device. However, for the generation of solid tumors the device was operated by high voltage ultrasound. The signal generator was connected to an RF amplifier (75A250, Amplifier Research, USA) and was monitored with an oscilloscope (TDS620B, Tektronix, USA). The two different multiwell designs were operated with different central frequencies. The square-shaped design was operated with frequencies of 1.96 MHz and 2.00 MHz and frequency modulation (also referred as linear sweeps) was set to 1 kHz rate and 50 kHz to 100 kHz span, respectively. The concave-shaped design was operated by a central frequency of 2.46 MHz and frequency modulation with 1 kHz rate and 100 kHz span. Actuation voltage ranged from 60 to 110 volts peak-to-peak measured with a
Chapter 2: Materials and Methods

10× attenuator probe (PM8927A, Philips, Netherlands). The device was integrated in a small table top cell incubator (Micro Galaxy, RS Biotech Laboratory Equipment, Scotland), and the temperature generated by the high voltage was regulated with a custom built temperature control system allowing temperature to maintain stable around 37°C during the entire experiments that could last up to 7 days with continuous ultrasound exposure.

2.5 Cell imaging in the microchip

NK cells and targets were labeled with different fluorescent molecules in order to be observed and distinguished during fluorescent microscopy. By time-lapse live cell imaging it was then possible to follow cells in the microwells and collect images continuously for several hours or even days.

Images in this Thesis were acquired with two different microscope systems: an inverted laser scanning confocal microscope (LCSM, Zeiss LSM 5 Pascal) equipped with a motorized stage, and an inverted bright-field microscope (Axiovert 40 CFL, Zeiss, Germany) equipped with a DSLR camera (Sony α77, Sony, Japan) and objective (Epiplan 5×/0.13, Zeiss, Germany). The Axiovert 40 CFL microscope was used only for bright-field imaging during the tumor formation experiments and mostly in experiments presented in Paper III. Fluorescent microscopy imaging was performed using a dichroic beam-splitter HFT488/543/633 and different combinations of excitation wavelengths and emission filters depending on the staining probes: calcein green (Ex: 488 nm/Em: BP505-530); calcein red-orange (Ex: 543 nm/Em: BP560-615); DDAO (Ex: 633 nm/Em: LP650).

In Paper IV, the trapping performance of the wedge-transducer device for adherent and non-adherent cells was investigated using a 2.5×/0.075 objective, allowing imaging of all 100 wells in the same field of view. For the cell cluster tracking and NK-mediated target cell killing experiments, a 10×/0.3 objective was used. All wells could be imaged in a time-lapse manner using the moving stage to form a 4×4 tile scan repeated every 6.8 ± 0.6 minutes. For cell cluster tracking the time-lapse imaging lasted 17 hours, while the NK killing experiments were performed for 4 hours (or 5 hours as in the experiment presented in Figure 8, and in Paper V). Imaging with the 10× objective was performed with the confocal pinhole opened to maximize detection of fluorescent light.

In Paper VI, high-resolution 3D confocal imaging of individual solid tumors was performed using a 40×/1.3 oil objective and pinhole adjusted according to the fluorescence signal in order to acquire the optimal image. Fluorescence imaging of the entire chip was performed with a 10×/0.3 objective using the moving stage to form a 4×4 tile scan. When time-lapse imaging of the tumors was performed, the 4×4 tile scan was repeated every 30 minutes.
Detector gain and brightness settings were optimized according to the intensity of the lasers and the staining in order to provide the best image outcome. Live cell imaging was performed in standard environmental cell culture conditions (37 °C and 5% CO₂) obtained by an environmental chamber integrated on the microscope. However, when imaging was performed while ultrasonic actuation, the temperature would slightly increase due to acoustic energy loss into heat. Therefore, to avoid overheating the cells, the temperature was measured on the chip and the thermostat of the environmental chamber was set to 33.5 ± 0.5 °C, leading to a stable temperature of 37 °C of the sample.
Chapter 3: Results

3.1 Cell culture in microenvironments

The initial idea involved the possibility of cells to grow in confined environments, in order to accomplish resolution of large numbers of individual cell behaviors in parallel. A proliferation study was required to evaluate the microwells as cell culture environments and investigate whether cells could survive and also sustain their physiological functions such as proliferation and in the case of NK cells, the ability of killing target cells. Viability and proliferation test showed that regardless the space limitation, nutrients and oxygen could diffuse to the bottom of the wells and cells were delivered the components needed for survival and growth. In addition, accumulation of cell-waste products did not appear to be toxic for cells within the microwells. Furthermore, toxicity of the materials was tested and any limitations were overcome after optimization of some microfabrication processes. Another concern involved whether the cells were indeed confined in the wells or were able to escape to adjacent wells. Cells being able to escape would limit the possibilities to follow and obtain information from individual cells for extended time periods. In order to explore whether the microwells can provide a healthy environment for living cells, several experiments were performed using 2 different cell lines. Both cell proliferation and confinement could be tested with the same set of experiments. This involved attempt of seeding single cells per well and following the events for up to 4 days. Since the cells are stochastically sediment, several wells were either empty or contained more than single cells.

For the small-sized multiwell microchips, observation of empty wells and the fact that their number remained constant or slightly increased over the examination time, supports the notion that cells could not transfer from one well to another once they were sediment. The small increase of ‘empty well’ numbers over the cultivation time was due to spontaneous death of single cells in some wells (since dead cells were not counted). However, the number of wells containing single cells the first day, significantly decreased suggesting cell proliferation. Once biocompatibility was ensured, other cell functions where tested in the wells. Specifically, the ability of natural killer cells to kill target cells or form inhibitory synapses was investigated and quantified (results are shown in Paper II at Figure 6). The results showed that both synapse formation
between NK and targets as well as NK cell-mediated cytotoxicity functions against targets are sustained in the multiwell microchip. These findings confirmed the feasibility of the idea thus promoted further research on the device development and more thorough investigation of NK functions at the single cell level.

### 3.2 Acoustic trapping in the multiwell chip

The ultrasonic manipulation method was tested for different parameters. First, in Paper I, we investigated the device performance for trapping 5 μm polyamide beads at single frequencies between 2.55 MHz and 2.65 MHz and frequency modulation with bandwidth of 100 kHz around each central frequency with 1 kHz sweeps. Simulations of the acoustic displacement field in the solid structure (silicon) and the acoustic pressure field in the fluid (i.e., the wells) were verified by the experiments and confirmed that the locations of the minima of pressure squared are approximately the same as the minima of the force potential in the wells.

For single frequencies applied, results showed an elongated shape of the pressure minimum that slightly differed in orientation and shape and from well to well. Furthermore, the pressure node patterns differentiated for each single frequency actuated. Thus, a frequency modulation around the central frequency with a rate of 1 kHz promoted an averaging effect of all the single-frequencies, leading to a uniform pressure node pattern located at approximately the center of each well. This allowed simultaneous aggregation of particles or cells in the center of each well assisting in the case of cells up to 100 cell-cell interactions.

Another parameter tested in Paper I was the temperature increase during ultrasonic actuation. As expected, due to electromechanical losses in the transducer and acoustic absorption in acoustically lossy materials such as the glue layers and PDMS frames, the temperature on the multiwell chip was slightly increased. The maximum temperature increase occurred for frequencies closest to the optimal chip resonance around 2.60 MHz, also corresponding to the optimal manipulation effect. By adjusting the external heating thermostat, the overall temperature on the chip was regulated to physiological range ensuring cell viability under ultrasonic manipulation.

Several cell viability tests were performed for even up to 72 hours of continuous ultrasound exposure, however we quantified the experiments for 12 hours. More specific, non-adherent 221 cells were stained and pipetted in the multiwell during ultrasonic actuation of $10 V_{pp}$ and frequency modulation of 100 kHz around a 2.60 MHz central frequency. 221 cells were trapped within the 100 wells and time-lapse tile-scan live cell imaging was performed. Figure 5 shows the tile reconstruction of 16 tile scans that compile the total well area of the multiwell chip, where 221 cells are shown in green (stained with green calcein-AM).
Images were analyzed and viability was quantified. Results confirmed that cellular viability was not compromised during the exposure period (shown in Figure 6A). Furthermore, extra-long experiments of continuous ultrasonic exposure (up to 72 hours) verified the proliferation and survival of cells for extended periods of time. Therefore, single cells were able to yield clustered clonal cell populations within the multiwell microplate (Figure 6B). In conclusion, the experiments and analysis confirmed that ultrasonic forces generated in our system are strong enough to retain the cells in the center of the wells over long time periods without negatively affecting their viability.
Figure 6. Viability study. (A) Calcein-labeled human B cells were seeded in the device and exposed to continuous ultrasound for a period of at least 12 hours. Images were scanned every 3 hours and viable cells were scored. In total, 4482 cells were counted. The results are compiled from three independent experiments and error bars are standard deviations of the mean. (B) A representative sequence of images showing a single trapped cell proliferating into a cluster of several cells while exposed to 67 hours of continuous ultrasound. The scale bar is 100 µm.

3.3 System improvement and applications

3.3.1 Trapping efficiency

The updated version of the wedge-transducer ultrasonic microdevice (as described in Figure 2) was developed to minimize complications related to device preparation and handling. The new design was used to address questions related to the device performance and consequently investigate cell functions through cell interactions.

Two different cell lines, the 221 (non-adherent) and 293T (adherent) were added in the microchip. The cells were allowed to sediment and adhere for approx. one hour before the experiment. The trapping dependency on actuation voltage for adherent and non-adherent cells was investigated by gradually increasing the ultrasonic actuation voltage from 0 to 10 V_{pp} while images were collected. Already at low voltage (<2 V_{pp}), the majority of non-adherent 221 cells aggregated to a central position, with a plateau of about 90% cells aggregated at voltages 3 V_{pp}. In contrast, even at higher voltages the majority of non-adherent cells were not aggregated, showing that the force of adhesion to the glass was stronger than the aggregation force delivered by the ultrasound. Additionally, 221 cells engaged in cell–cell contact, with adherent 293T cells
outside the trapping position, often remaining attached to the 293T cells despite a gradual amplitude increase in the ultrasonic actuation. Occasionally, we observed (but not quantified) that conjugates of the two different cell types would detach from the glass substrate and move to the trapping position, supporting the notion that ultrasonic forces are weaker or comparable to cell-cell forces.

### 3.3.2 Synchronized cell aggregation

To determine whether rapid, simultaneous aggregation of cells in all 100 wells was possible using ultrasonic actuation, non-adherent cells were seeded, left to settle and subsequently exposed to 10 V_{pp} actuation voltage. This resulted in aggregation of the majority of cells in all 100 wells within minutes. Next, the long-term stability of the cell positioning in the microwells with and without ultrasonic exposure was studied. A mixture of human peripheral blood NK and 221 cells (approx. 10–20 cells per well) were seeded in the chip and allowed to settle at the bottom of the well. The ultrasound was turned on and cell aggregates were formed in each well. After completing a 4×4 tile scan covering the whole chip (approx. 7 min) the ultrasound was either kept on or switched off. Cell aggregates were tracked for 17 hours, and the distance between the center of the aggregate and the center of the well was calculated for each time point. The positions of aggregates maintained under continuous ultrasound exposure were offset from the center of the well by on average 35–40 μm, and displaced on average 5 μm from that position over the 17 hour long experiment. In contrast, when ultrasound was turned off, the aggregates gradually moved away from the initial trapping position. The timing and direction of this movement varied between aggregates in the different wells, but on average the aggregates were displaced 50 μm from the initial position during the 17-hour movie. Hence, when ultrasound was kept on, the majority of cell clusters were retained within approx. 10 μm of the initial central trapping position, giving the possibility to acquire multiple high-resolution time-lapse movies in parallel.

### 3.3.3 Synapse formation under ultrasonic actuation

Besides biocompatibility of the device other tests should be performed related to other important cell functions and whether they are maintained under the effect of ultrasound. To test the ultrasound effect on the immune synapse formation, we used the NK cell line YTS- KIR2DL1 and 221 cells transfected to express MHC HLA-Cw6 (cognate ligand to KIR2DL1) tagged with GFP (221/Cw6-GFP). The robust positioning of the ultrasound was used to study NK–target cell conjugates by high-resolution 3D confocal microscopy over time. Consistent with formation of inhibitory immune synapses, the HLA-Cw6-GFP protein accumulated at the intercellular contacts between these two cell types. Among 53 immune synapses formed in 33 separate NK–target cell aggregates (≥1 synapse/aggregate), we observed no target cell death during continuous ultrasound exposure for at least two hours. An example of a single YTS cell attached to five 221 cells is presented in Figure 7 in a time lapse sequence. These experiments show that the positioning of cells induced by
ultrasound-mediated aggregation facilitates high-resolution imaging of cell–cell contacts without affecting important cellular functions such as protein accumulation at the synapse or NK cell inhibition.

![Figure 7](image)

**Figure 7.** Time-lapse recordings of the inhibitory interaction between a single NK cell (YTS KIR2DL cell line) and five target cells (721.221/Cw6-GFP). (A) Bright field image of the interaction indicating the morphology of the cells. White arrows point out the target cells. At $t = 100$ min the NK cell has divided into two NK cells. (B) Merged images of bright field and green channel indicating the accumulation of GFP protein (white arrows) in the interface between each target cell and the NK cell (the immune synapse). At $t = 100$ min, two of the synapses (of two target cells) are shown to have divided (red arrows) at the site of the NK cell division (GFP gap). (C) False-color coding of the green fluorescence clustering at the immune synapse. Here, the divided synapses are highlighted with black arrows.

### 3.3.4 Ultrasound-assisted NK-target cell interactions

Ultrasoundic manipulation experiments performed with primary IL-2 activated NK cells and 221 target cells, confirmed that NK cells had the ability to kill target cells under the effect of ultrasound. Repetition of the experiments, analysis and quantification of the results revealed a functional heterogeneity within the NK cell population. In detail, first 221 cells and then NK cells were seeded in the multiwell microdevice and allowed to sediment. Cells were seeded to achieve a distribution of approximately one NK cell and 2–4 target cells in
each well. In total 402 NK cells and 1106 target cells were scored in various effector–target cell combinations. Cell–cell contact was subsequently induced by ultrasound and the interactions in each well were imaged every approx. 7 minutes for four hours. As shown in Paper IV in Figure 4, the chance of target cell death increased with the total number of NK cells present in each well. Results showed that death occurred in 13% of the wells without NK cells, 64% in wells containing 1 NK cell, 77% with 2 NK cells and approx. 90% with 3 or 4 NK cells. The significant increase in target cell death in the presence of NK cells clearly shows that NK cells could kill target cells in the assay. However, not all NK cells were capable of killing target cells, since no killing was observed in 36% of the wells containing single NK cells and at least one target cell. The increase in killing with an increasing number of NK cells shows that the more NK cells present, the more likely it is to have at least one that displays cytotoxic response. Examples of the three different types of NK cell behavior against 221 cells over a 5 hour period is shown in Figure 8. However, this experiment is not included in the quantification described above.

Next, we investigated the killing potential of individual NK cells, and therefore we focused on wells that contained single NK cells with 3–10 target cells. Wells with 1–2 targets were excluded from the analysis since the numbers of targets were considered too low to assess the killing capacity of the NK cells. This analysis showed that there was a heterogeneity in individual cells’ capacity to kill target cells (Table 2).

Table 2. Killing capacity of individual NK cells. Light and dark grey areas show the number of events scored for NK cells with low or high cytotoxicity, respectively

<table>
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<th>Number of kills</th>
<th>0</th>
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<th>2</th>
<th>3</th>
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<td>13</td>
<td>7</td>
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</tr>
<tr>
<td>4</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>8-10</td>
<td>2</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* one target cell divided

The killing efficiency of individual NK cells varied significantly, with some cells (18/76) being able to kill three or more target cells in a consecutive manner during the four-hour long assay, while other NK cells were observed not to kill at all (22/76). When we quantified killing over time, we found that NK cells killing up to two target cells showed a frequency of one kill every approx. 1–3 h, while serial killers (here defined as killing three or more targets during the assay time of four hours) killed a target every approx. 0.5–1 h. Consequently, IL-2-activated human primary NK cells show a great heterogeneity in their
cytotoxic response with a significant fraction of inactive cells and some serial killers capable of killing several targets within a time frame of a few hours. Next, we investigated how the responsiveness of individual NK cells depended on the level of stimulatory input from target cells. Furthermore, during the evaluation of killing in wells containing single NK cells and various numbers of targets, we observed that the chance of NK cell killing was 43% when one target cell was present, and increased to 64% with two target cells. When all cases with more than two target cells were pooled, a plateau was reached with killing occurring in 69% (weighted mean value) of the cases. Statistical analysis showed that the difference observed going from one to several target cells was significant (p<0.05), also when the level of spontaneous cell death (5%) was considered. When we analyzed only the NK cells that had committed a first kill we made a similar observation, i.e. if there was more than one target cell still alive in the well it was more likely that a second kill would occur. This difference was also statistically significant showing killing efficiency increasing from 28% to 60%. Therefore, NK cytotoxicity was more likely to occur if several live target cells were present in the NK–target cell aggregate.
Figure 8. Time-lapse image sequences of three parallel events among the 100 wells of the ultrasonic microplate in a 5 h cytotoxic assay. NK cells are shown in orange (red-orange calcein-AM), living target cells are shown in green (green calcein-AM) and dead target cells in red (far-red DDAO-SE). (A) Continuous interaction of a non-active NK cell (non-killer) with four to five targets (target cell division at t = 132 min) during the entire assay does not result to any NK cell mediated death. (B) Normal killer induces killing at t = 19 and 57 min, but remains inactive (although in contact with living target) for the remaining experiment. (C) Serial killer eliminating all four target cells within 75 min.
3.4 Generation and categorization of tumors

As several studies support the necessity of three-dimensional cell culture system in vitro, we investigated the possibility to generate solid multicellular structures in the ultrasonic multiwell microdevice. We hypothesized that acoustic radiation forces in the chip were sufficient to assist formation of solid tumors when applied on cells with tendency to form strong intercellular contacts. The epithelial cell line HepG2 was chosen due to some important characteristics. As mentioned previously, HepG2 cell line is derived from hepatocellular carcinoma i.e. one of the most common reasons of mortality worldwide. Thus, HepG2 have been widely used either as monolayers or as multicellular cultures to investigate chemotherapy drug toxicity on HCC cells, or as models of liver cells to explore drug-induced liver toxicity. Furthermore, HepG2 cell line has been tested previously and verified susceptibility to NK cell-mediated killing\textsuperscript{108}, therefore the formed tumors could be used consequently as targets to investigate NK cell properties such as infiltration and cytotoxicity against solid tumors.

HepG2 cells were trypsinized and were washed twice in RPMI-1640. After HepG2 were counted and prepared with the desired concentration, cells were seeded in the multiwell microplate in the ultrasonic ring-transducer device. The device was then connected to the signal generator-RF amplifier and was placed under the microscope. When cells were sediment but not allowed to adhere, we increased the voltage so that cells were trapped in the pressure nodes as described previously. By monitoring the temperature during trapping, the voltage was set to the highest possible that would not increase the heat more than 37\(^\circ\)C. Subsequently the device was placed in the incubator-temperature regulation system. Results confirmed that cells were able to survive under high voltage ultrasound for the entire experiments (up to 8 days) as long as the overall temperature on the cells would not exceed 37\(^\circ\)C.

Solid tumors were defined as highly compact structures with large intercellular bindings between adjacent cells. In addition, characteristic sharp tumor boundaries were clearly observed in both dark field (Figure 9A) and bright field (Figure 9B) images of solid tumors.
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Figure 9. Characteristic morphology of solid tumors composed by HepG2 cells. (A) Dark field image obtained by a $10\times/0.3$ objective. (B) High-resolution bright field image of a relative small size tumors. The image was acquired with a $40\times/1.3$ oil objective and the image plane is located approximately in the center of the tumor.

Solid tumors occurred in most of the 100 wells (as shown in Figure 10A). However, the results varied depending on the duration of each experiments, the well geometry, the voltage actuation amplitude, the amount of oil or gel used on the device and other processes that could lead to alteration of the voltage applied on the device. But also we believe it is related to cells condition prior to experiments and the duration of trypsinization. To validate the effect of ultrasound on tumor formation, we performed several control tests where cells were seeded in the microplate and were cultured in the same conditions without being exposed to ultrasound. Results verified the necessity of ultrasound to generate solid tumors, considering that they were not formed in any of the control experiments. In contrary, all cells grew irregularly in monolayers, adhered in the glass bottom covering the whole well surface and kept wide intercellular spaces (Figure 10B). These results agree with the morphological characterization of monolayer HepG2 structures described in reference\(^7\).

The Figures 10C and 10D display zoomed-in images of an ultrasound-formed solid tumor and a monolayer culture in the control experiment respectively. Another interesting observation related to the culture morphology involves the staining effect of calcein on solid tumors versus monolayers as shown in Figures 10A and 10C and Figure 11. Due to the tight intercellular contacts of cells in solid tumors, penetration of calcein is limited to approximately three cell layers. In contrary, monolayers are stained uniformly due to direct expose
of all cells to calcein. Hence, we have utilized this characteristic property of calcein limited influx to distinguish 3D cultures than monolayers on calcein-stained samples.

Figure 10. Solid tumors formed in the ring-transducer device and a control experiment. (A) Green calcein-AM stained HepG2 cell tumors after 7 days of continuous ultrasound exposure. (B) Control experiment of a 7-day culture without ultrasound, cells grow in monolayers. (C) High-resolution image showing a solid 3D tumor in one of the 100 wells of (A). (D) High-resolution image of a well in the control experiment were cells grow disorganized and in monolayers.

The tumors generated by the ultrasonic manipulation system were visually categorized according to the morphology of the 3D structures. As the optimum category was defined the first (I) where the ratio of the height divided to the tumor diameter was approximately one, indicating a more spheroid structure.
A high-resolution image of a solid tumor that belongs to the first category is shown in Figure 11.

![Figure 11. A solid tumor of HepG2 cells stained with green calcein-AM. In the XY cross-section of the tumor the cells are shown to form a compact structure of maximum diameter approx. 65 μm. In the vertical cross-section of the tumor the height is approx. 55 μm.](image)

The category (II) is defined as structures of height/area <1 and they contain a small region of monolayer in the perimeter of the 3D structure. In category (III) the surrounding monolayer area is larger and the height of the 3D structure is noticeably smaller. The forth category (IV) is defined as a cell monolayer that in some occasions contains small areas with overlapping cells. Finally, the last category (V) corresponds to poor trapping conditions and is characterized by three or more separated monolayers and/or tumors. Examples of the different solid tumor categories are presented in Figure 12A. Bright field microscopy images were analyzed and the results were quantified. Examples and quantification of the different categories are shown in Figure 12B.
Figure 12. (A) The five different categories of the solid tumor morphology (I to V). (B) Quantification and sorting of the experiments according to the different categories. The average percentage of category I and II for the first four columns (ultrasound exposure without following incubation) is 85% meaning that the efficiency of 3D cell culture formation is 85 wells out of the 100 wells on average for the multiwell microplate. Exposure times from 15 min to 4 hours was insufficient to yield the optimum categories I, II. In contrary, the highest efficiency of the system occurred for exposure times of 2 days or longer. Subsequent incubation of the cultures after ultrasonic exposure would shift the category distribution from I towards II and III as the tumors expanded.
3.5 Imaging of NK-tumor interactions

3.5.1 Natural killer cell versus solid tumors

After the tumor assembly and formation experiments, the multiwell microchip was removed from the ultrasonic ring-transducer device and was placed in the cell incubator for approximately 2 days. Natural killer cells were isolated from whole blood (see materials and methods chapter) and cultivated for at least 7 days prior to experiments. Subsequently, NK cells and tumors were stained and the NK cells were seeded in the chip. The multiwell microchip was placed in the aluminum holder and under the microscope, followed by confocal fluorescence imaging either in a time-lapse tile-scan manner (image sequencing of the entire chip) or 3D high-resolution imaging of individual tumors. Four experiments were performed and the three were followed for long time periods even up to 11 days after NK cell seeding. Therefore both imaging approaches in different combinations were feasible for each of the experimental sets.

3.5.2 Time-lapse tile-scan imaging of NK-tumor conjugates

Time-lapse imaging of natural killers interacting with solid tumors revealed as expected, a dependency of the tumor outcome on the number of NK cells. Quantification of the analyzed images confirmed that the number of NK cells on the tumor and the tumor volume define if it would grow, shrink or remain relative unchanged. The balance between tumor growth and NK-mediated killing was not sustained for extended times and eventually the tumor would either escape NK invasion and grow, or would be suppressed and finally eliminated. This was verified from one experiment that was followed and analyzed for a time period of 5 days. Among 100 wells the 15 were excluded either due to bad trapping that allowed tumors to grow on the well sides, or they empty or unclean. The remaining 85 wells were analyzed and the tumor outcomes after 22 hours and 120 hours were scored. Among 85 solid tumors, 35 shrunk, 30 remained unchanged and 20 increased their size after 22 hours of NK seeding. Scoring of the same wells 120 hours after seeding revealed that all 35 shrinking tumors were dead and disaggregated, 21 of the 30 unchanged were also disaggregated while the rest 9 tumors had grown. Finally among the 20 tumors increasing their size in the first 22 hours, 18 had grown further, one had shrunk and one was had returned to the initial size (before or at the time of NK cell seeding). Image sequences showing the outcome of two different size tumors from the experiment is presented in Figure 13.

In addition, the tumors were classified to the five different categories explained previously and the number of NK cells on the tumors was estimated. Each tumor area was marked and the bright field mean intensity of the tumor and the well background was measured in ImageJ. As ‘intensity’ we refer to the
Imaging of NK- tumor interactions

subtracted value from the background intensity. Therefore, a high intensity value corresponds to thicker tumor.

Finally, we calculated the ratio of the number of NK cells on each tumor versus the area of each tumor multiplied with the normalized tumor intensity that is estimated. The value of (Area*Intensity) is an estimate of the tumor volume. Therefore, the value of the number of NK cells per tumor volume is directly related to the tumor fate. As expected, plotting the (No of NK cells/(Area*Norm Intensity)) for each category versus the tumor outcome (decrease, constant, increase) revealed a trend where the decrease of the value was proportional to tumor growth (Paper VI, Figure 7 and Figure 8). The results also revealed a trend between the different categories according to their susceptibility on NK cell killing, however more data are needed to make significant conclusions especially in order to compare susceptibility to NK cell cytotoxicity of solid tumors versus monolayers.

3.5.3 High-resolution imaging of NK cells fighting tumors

One of the advantages of the multiwell microplate as a cell culture system is the ability to perform high-resolution imaging directly in the wells. Cell-cell interactions either they are formed within the confined areas of the small size multiwell chips, or due to ultrasonic manipulation in the 300-350 μm multiwell chips, are able to be imaged in high-resolution-time-lapse manner for several hours. This is accomplished by the limited migration region of cells or cell clusters within the imaging field of view. In this project we have observed in high-resolution the accumulation of MHC class I proteins tagged with GFP in the inhibitory synapses between NK cells (YTS/ KIR2DL1) and 721.221/Cw6-GFP. In addition, we have been able to perform high-resolution imaging of HepG2 solid tumors formed by our ultrasonic manipulation system and record the internal tumor structure. A small size tumor stained with green calcein-AM and imaged in high-resolution is presented in Figure 11. On the left, we display a cross section of the tumor close to the well bottom and on the right a vertical section. An example of a bright field high-resolution XY image showing structures within the solid tumor is presented in Figure 9B.
Figure 13. Image sequences of NK cells against two solid tumors over a 124-hour time period. (A) Fluorescence (combined with bright field) images of NK cells shown in orange and a solid tumor shown in green (alive cancer cells) and red (dead cancer cells). At time 0 h very few NK cells are in contact with the tumor. At t =1 h more NK cells are interacting with the tumor. At t= 22 h the perimeter of the spheroid has altered by dead cancer cells (red) detached from the tumor (green intensity has decreased due to NK mediated death and bleaching). At t= 51 h extra green calcein-AM is added to the chip, staining the cells that are alive (both NK cells and cancer cells). At t= 124 h the tumor is dissolved after calcein was added. The NK cell morphology suggests that the green cells are NK cells and the tumor cells are dead. (B) Bright field image sequence as (A) with an extra image at t= 120h showing the spheroid before calcein was added. At that time the tumor has already started to disaggregate. (C) A different well of the same experiment as (A) showing a larger tumor that continues to grow over time despite the presence of NK cells. (D) Image sequence as described in (B) showing in bright field the development of the tumor in 124 h.

Subsequently to the investigation and categorization of the solid tumors we have also studied the behavior of natural killer cells against these tumors. Besides the quantification of NK cell mediated tumor killing described previously, we have in addition performed high-resolution imaging and observed individual tumors invaded by NK cells. Results showed penetration of NK cells in solid tumors, usually by direct attachment on the tumor cell followed by killing of the cell and consequently infiltration within the tumor.
Identification of calcein after approximately 24 hours was not feasible due to bleaching and due to calcein limited penetration through cell junctions in the tumors. Therefore, when the aim was to identify the condition of the central area of tumors after several days, additional calcein staining was performed. In contrary, far-red DDAO stain was still present even after several days, so no further staining was needed. Images of several solid tumors revealed intact smaller tumor structures underneath the dead cell layers (shown in red) supporting the notion of NK cell killing from the surface towards the center. An example is presented in Figure 14 that is a high-resolution image of the same tumor in Figure 13A at time point 51 h. Figure 14C display a XY slice of the tumor. Figures A and D display XZ and YZ tumor cross sections, respectively. Figure 14B display a bright field high-resolution image of the tumor.

![Image of tumor cross sections](image)

*Figure 14. Solid tumor cross sections of high-resolution confocal images of the spheroid presented in Figure 13A at time point 51 h. The cells that are alive are shown in green and dead cells in red (A) a XZ cross section, (B) bright field image of the spheroid, (C) the horizontal plane XY approximately 25 μm from the well bottom and (D) a YZ cross section.*
Furthermore, in high-resolution imaging NK cells show the ability to stretch upon attachment to a solid tumor as shown in a 3D reconstructed high-resolution image in Figure 15, where two NK cells (shown in orange) adhere on the surface of a tumor spheroid. Although NK cells seem to attack the tumor from the periphery and inwards, occasionally we observed events of NK cell penetration in a central region of intact solid tumors suggesting the ability of some NK cells to infiltrate through the tumor cell junctions.

Figure 15. Natural killer cell adherence to a tumor. (A) High-resolution confocal 3D image of a solid tumor spheroid (stained with green calcein-AM) and two natural killer cells (stained with red-orange calcein-AM).

Figures 16 and 17 present a solid tumor of approx. 100 μm in diameter that has been exposed to NK cells. Images were acquired after 2.25 and 19.5 hours after NK seeding, respectively. Figure 16C shows a horizontal cross section of the tumor at about 10 μm above the well surface. NK cells are attached on the tumor surface close to the bottom of the well. Yet, a single NK cell has penetrated the glass-tumor adherence surface and is located in the center, infiltrating the tumor from bellow. This is confirmed by the YZ and XZ cross sections of the spheroid in Figure 16B and 16C, respectively. The phenomenon of NK cell accumulation and infiltration through the adherent site of the tumor with the glass has been frequently observed, suggesting a better accessibility for NK cells to penetrate the tumor. The Figure 16B presents a bright-field high-resolution image of the spheroid. The spheroid is shown to be mostly intact and maintains the characteristic boundary on the upright corner, however killing has already initiated and cells are shown to detach from the tumor. The follow up imaging of the tumor is performed 17 hours later and is presented in Figure 17. A horizontal cross sections is shown in Figure 17C where NK cells have penetrated and migrated further in the tumor from the center bottom area and upwards. The NK position is also confirmed by the vertical cross sections shown in Figures 17A and 17D. However killing had mostly occurred at the periphery of the tumor as shown both in the fluorescent images in red color, but also in the bright field image that shows disaggregation of dead cells from the tumor.
Figure 16. Solid tumor spheroid cultured with NK cells for 2.25 hours. In the fluorescent images, NK cells are shown in orange (red-orange calcein-AM) and the tumor is labelled in green (green calcein-AM). (A) A vertical XZ cross-section of the tumor, (B) a bright field image of the tumor spheroid showing that the tumor is mainly intact but NK cell mediated killing has initiated in the periphery, (C) Horizontal (XY) cross section of the spheroid and (D) a vertical (YZ) cross section of the spheroid. The combination of the three different planes validate the presence of the NK cells in the middle of the low tumor surface.
Figure 17. Follow up image of the tumor presented in Figure 16. An overview of the tumor 19.5 hours after NK cells were seeded in the chip, clearly shows killing in the periphery of the tumor (dead cells are shown in red) and NK cells (orange color) penetrating within the tumor (A,C,D tumor cross sections as described previously). (B) The bright field image of the spheroid also indicates tumor cell death in the periphery and dead cell disaggregation.
Chapter 4: Discussion

4.1 Method development

4.1.1 Multiwell microchips

The aim of this project was to downscale the conventional cell culture 96-well plates to a culture system that allows cell investigation in the level of individual cells. In addition, the limitations of conventional techniques for live cell imaging over long time periods, led to the development of the multiwell microplate. The initial design consisted of squared shape wells of 50-80 μm size, and the question arise was about the feasibility of the idea and whether the cells could survive within engineered confined microenvironments. The potential risk for the cells viability was to not get enough nutrients and gas caused by a non-stirred medium environment in wells. Furthermore, accumulation of the cell wastes inside the wells could damage the cells. Basic viability experiments verified the biocompatibility of the microchip since cells did not only survive but also proliferated within the wells. However, later experiments with the second generation custom made multiwell chips verified the significance of some fabrication steps on biocompatibility.

For single cell analytical systems besides biocompatibility it is also crucial to maintain individual cells isolated from other cells located in adjacent wells. Experiments confirmed the constriction of cells within the wells, which supported the relevance of the scheduled applications of the device. Nevertheless, statistical data showed a short percentage of cells migrating to other wells. This could be due to a human visual error, insufficient removal of the excess cells after seeding or overfilling with cells when cultured in the chip for several days.

One issue to overcome during preparation of the chip for cell seeding, was the removal of air bubbles that were trapped in the bottom of the wells and could inhibit the free passage of the medium to fill the wells. A simple solution to resolve this problem was utilizing a vacuum environment. Moreover it was important to sterilize the chip and completely remove the residues of the ethanol and water and replace them with the medium before the cells were seeded in the wells. Several other challenges related to device cleaning, priming with medium, leakage and overall handling were in general achieved.
Since the applications of the device were aimed towards NK-target cell interactions upon contact, the confined micowells could ensure at some point the desired cell collision. Still, the exact time of interaction could not be determined and could vary from well to well. Although the small wells have the benefit of a large number of individual wells thus observable interaction events, they have a limited capacity of cells per well therefore prohibits culturing of cells up to several days. The well capacity challenge and the synchronization of cell collisions were addressed with the development with the ultrasonic cell manipulation system.

4.1.2 Ultrasonic multiwell device study

Several studies in the field of acoustics in microfluidic systems has shown particle trapping in microchannels accomplished by acoustic radiation forces due to acoustic resonance in the microchannels\textsuperscript{62,67,97,113}. The theory suggested that implementation of the multiwell microplate in an acoustic field under suitable conditions, could lead to acoustic resonances formed in each one of the wells. Therefore, the ultrasonic manipulation method could expand from particle trapping in single channels to an array of wells. Initial investigation with beads confirmed the formulation of acoustic radiation forces within each well of the multiwell microplates when the geometry of the wells matched the ultrasonic frequency applied on the chip. Specifically, in order to create a single pressure node within each well, the size of the well should correspond to half wavelength of the ultrasonic frequency applied. This led to fabrication and usage of the 300–350 µm wells, which allowed cell culturing for several days, even weeks. The method was examined with cells and confirmed the ability of the device to trap cells in the pressure node of each well. This allowed a synchronization of cell-cell interactions in up to 100 wells.

The more obvious effects of ultrasound manipulation is the primary and secondary radiation force acting upon particles in the fluid. However and important effect is the acoustic streaming which is always present. This effect dominates particles typically smaller than 1 µm, however the effect could be of importance when dealing with cells. Although acoustic streaming can assist cell trapping, when high voltages are applied, streaming could lead to levitation of the aggregate and flash it out of the well. Acoustic streaming could be decreased by frequency modulation\textsuperscript{114,115}.

Although the device was functional according to the theory, several other challenges had to overcome related to device handling and usage. Particularly leakage was a major drawback at the early stage of the device development. This issue was easily resolved after plasma bonding the PDMS frame on the chip. In addition, a more robust transducer design including the device holder led, to an easier overall device performance and handling.
In this project two ultrasonic manipulation designs were used. First, the wedge-transducer designed previously in the group \(^\text{112}\), was upgraded to carry more robust characteristics, and subsequently, the ring-transducer was designed and employed. High voltage ultrasound actuation is possible for both device designs; nevertheless it was applied mainly on the ring-transducer device. The wedge-transducer was operated at low voltages and was efficient enough to induce cell contacts for long time periods of microscopic imaging, however it could not operate in humid environments like a cell incubator. Therefore, for the solid tumor formation project which required long incubation periods of continuous ultrasonic actuation, the ring-transducer device was used.

An essential limitation of the ring-transducer compared the wedge-transducer device is the inability to perform high-resolution imaging directly on the device. In contrary time-lapse tile-scan imaging can be sufficiently performed. This imaging disadvantage of the ring-transducer was not an issue in the tumor formation project since the chip was removed from the device and was placed in a holder and maintained in the ordinary cell incubator prior to the NK cell experiments. In addition, ultrasonic actuation was not necessary while imaging, since tumors were already formed before the interaction experiments. Additionally, the NK cell collision with the tumors was always achieved due to the large size of tumors and high number of NK cells in the wells. For future applications it would be beneficial to upgrade the system so that high-resolution imaging can be performed during ultrasonic actuation.

**Method eligibility for biological research**

Since biocompatibility of the silicon-glass chips was confirmed in the early stage of the project, the effect of ultrasound on cell viability was to be examined. Previous viability and proliferation tests were performed in our group on adherent monkey kidney cells (COS-7) at different ultrasound exposure times in microchannel chips. Results suggested that in the specific cell type, the ultrasound exposure on cells did not cause harm but in contrary showed to be beneficial for cell proliferation that the control experiments \(^{116,117}\). Experiments on the multiwell chips verified the biocompatibility of the system when temperature was sustained in cell physiological levels. Besides cell growth, it is important that the cell functions maintained when the cells are exposed to ultrasound. Several studies have been performed where different cell types and cell functions have been investigated after short-term ultrasound (seconds to minutes) \(^{73,75,118}\). In previous studies, other groups have measured the forces required to pull apart two conjugated cells to be in the range of approx. 100 nN \(^{119}\). Ultrasonic radiation forces in devices similar to ours have previously been measured to be in the order of 10–1000 pN \(^{113,120-122}\). In Paper III, Ohlin et. al., measured the ultrasonic forces in the multiwell microplate to be up to 50 pN. Therefore, we trust that although the forces are significant for
generating cell–cell contacts, yet not strong enough to interfere with biological forces responsible for, e.g., cell–cell or cell–substrate adhesion.

Additionally, we studied the functional properties of NK cells, such as the ability of NK cells to form immune synapses and to selectively kill tumor cells during long-term ultrasound exposure (up to several hours). Our results clearly confirmed besides cell growth, also the ability of NK cells to maintain their characteristic properties and either kill when they are triggered, or discharge the targets when they carry and present MHC class I inhibition molecules on their surface.

**Method limitations**

Limitations of the method are mostly related to imaging. A common problem that we encounter during the imaging experiments is bleaching of the calcein dyes. Calcein staining appears to be very stable for the first 6-8 hours, however over time tends to lose its fluorescence. One explanation is that since calcein is an organic dye possibly becomes metabolized in the cells and loses its fluorescence or slowly leaks out through the cell membrane. Another reason is photobleaching during laser exposures while imaging. Since our system is designed for short and long term experiments, we lose partly its potential due to these imaging limitations. A solution could be the additional staining directly in the chip, however this limits the possibility to distinguish among different cells types. Nevertheless, occasionally it is possible to resolve the different cell types due to the different morphological characteristics. Since calcein does not penetrate through the first three cell layers in the core of a tumor, another limitation is to distinguish infiltration and killing within the tumors. Other imaging limitations are related to the low temporal resolution due to the microscope scanning performance. For example, in the Z-scan 3D imaging of the solid tumors, a slow scanning performance has caused loss of information about NK cell migration within the tumors. However, this limitation is not directly related to the device.

Other limitations exist in the process of removing and replacing new medium either for subsequent culture or for the labeling of the tumors. In most cases the tumors are attached in the bottom of the wells, which can be considered as a drawback on the sense of not always display the typical spheroid shape. However, adherence helps maintaining the tumors in the wells without flashing them during medium replacement. For example, in some experiments we had accomplished tumor aggregates to levitate within most wells allowing them to grow into the typical spheroid shape, however we lost many of them during staining that requires a few resuspension steps.
4.2 Natural killer cell study

The main biological target of investigation on the present study was the NK cells and their functions against individual target cells and solid tumors. Many studies about NK cells have been performed since they were first discovered in 1970s and several of their functions have been decoded, however there are many processes that are still unknown regarding the killing mechanisms against virus-infected cells and tumors cells. In order to identify some of these processes and subsequently understand the pathways of the immune system against human disease it is crucial to understand these mechanisms by which the NK cells interact with targets.

Conventional methods of investigating NK cell properties and functions (such as 51Cr release cytotoxicity assay) are based on bulk averages that hide information related to characteristics of individual NK cells. High NK heterogeneity has been shown by experiments performed on human\textsuperscript{123,124} and mouse \textsuperscript{125} NK cells, indicating the need of more efficient, high throughput and well controlled techniques for single cell analysis. With the multiwell microplate designs (i.e., the small well chips and the ultrasonic devices) we can induce NK-target cell interactions in parallel. The method allows imaging of isolated events within the individual wells. Thus, we are able to observe and record large numbers of individual interactions between NK cells and target cells and the resulting NK responses within a specific time frame depending on the experiment.

By screening wells containing IL-2 activated human peripheral blood NK cells and tumor target cells, we quantified the cytotoxic response of individual NK cells and observed a significant heterogeneity. Despite several days of activation, only a fraction of the NK cells killed target cells in the four hour long assay. Among the NK cells that did kill we found a sub-population that responded strongly by killing several target cells each. Such ‘serial killers’ have been described previously\textsuperscript{123,126}, but so far it is unknown if these cells represent a particular subpopulation that could be identified by specific molecular markers. Another interesting information achieved with our method was that individual NK cells were more prone to kill if they were interacting with several target cells. This could be due to a heterogeneity in susceptibility of target cells to NK induced killing. Another interpretation is that NK cell responsiveness could be increased when receiving stimulatory input from several target cells simultaneously. This remains to be answered in future work by testing this behavior of NK cells against different cell lines with more controlled experimental conditions. Yamanaka et al., has also used similar multiwell chips to generate NK-cancer interactions at the single cell level for subsequent investigation of NK mediated lysis and whether it is correlated to NK secretory activity. In their study they also observed a heterogeneity within an IL-2
activated NK cell population, but in addition they have resolve cytokine secretion. This secretion was correlated with reduce motility during NK contact to targets, but not with NK cytolitic activity\textsuperscript{127}.

In our ultrasonic manipulation system, besides inducing synchronized cell-cell contact and thus initiation of the interaction, we can also maintain the cell conjugates at a position not deviating more than 10 μm in each well allowing high-resolution confocal imaging. Therefore, by high-resolution time-lapse imaging at several positions, parallel NK-target cell interactions could be studied in detail. Since current high-resolution imaging techniques are limited by the tendency of cells to migrate or drift away from the imaging area (field of view), we provide a useful tool for addressing this challenge.

The last section of this Thesis was related to the NK cell activity when they are co-cultured with solid tumors. In this part of the investigation the NK cells were allowed to freely migrate towards the tumor, since ultrasound was not applied at any time after NK cells were seeded in the wells. The reasons for not inducing NK-tumor contacts via ultrasonic forces was first that NK cell numbers per well were significantly higher than single cells (however still countable). In addition, due to the large tumor sizes occupying a vast area of each well, interactions were expected to occur within minutes. Furthermore, due to the long duration of the experiments, we did not necessarily require synchronization of the interactions or high temporal resolution. Nevertheless, a future experimental approach could be to perform heterogeneity tests of individual NK cells behavior against solid tumors, therefore a gentle ultrasonic radiation force could be preferred to induce interaction.

## 4.3 Significance of the 3D-tumor project for future applications

In order to better recreate the three-dimensional microenvironment of solid tumors, we need to mimic the 3D structure of the developing tumor. 2D cultures that are commonly used are inadequate to recreate this microenvironment and therefore the outcomes of the experiments can be insufficient. However, there are few reported methods for the controlled formation of many 3D spheroids in parallel\textsuperscript{77}. Therefore, as the necessity of three-dimensional cell culture systems \textit{in vitro} is considerably increasing for various cell applications, we investigated the possibility to generate solid multicellular structures (i.e., tumors) in the ultrasonic multiwell microdevice. We hypothesized that acoustic radiation forces in the chip were sufficient to assist formation of solid tumors when applied on cells with tendency to form strong intercellular contacts. The epithelial cell line HepG2 was chosen for practical reasons, but also because it fulfilled the requirements for this project since they can form solid tumors and they are susceptible to NK cell-mediated
Significance of the 3D-tumor project for future applications

killing. The important characteristics of HepG2 cells and the significance of our methodology to investigate in the future these cells, is described below in this section.

As mentioned previously, HepG2 cell line is derived from hepatocellular carcinoma i.e. one of the most common reasons of mortality worldwide. Therefore, HepG2 have been widely used either as monolayers or as multicellular cultures to investigate chemotherapy drug toxicity on HCC cells, or as models of liver cells to explore drug-induced liver toxicity. In more detail, HepG2 cell line has been tested for several extracellular matrix protein expression in 2D and 3D proteins revealing the privilege of 3D cultures systems. Other studies though suggest a functionality difference between monolayers and multicellular cultures due to findings showing upregulation of structural genes such as extracellular matrix, cytoskeleton and adhesion molecules in monolayers while 3D cultures show upregulation of synthetic and metabolic genes. Furthermore, enhanced liver-like functions are shown to maintain in 3D spheroid cultures rather than monolayers indicating the necessity of preserving the physical environment for hepatic cellular functions for efforts on liver-tissue engineering research.

Alternative studies have also characterized survival advantages of 3D HepG2 cultures versus 2D when treated with different chemotherapy drugs such as cisplatin, 5-fluorouracil and Doxorubicin. The 2D and 3D HepG2 cultures were investigated on cell cycle progression and apoptosis as well as inhibition of cell proliferation. Drug resistance and protein expression tests revealed that cells from the multicellular cultures showed increased integrity after treatment than the cells grown in monolayers. This findings can suggest failure of prediction of drug resistance is partly due to results from 2D culture systems. Since the liver is the principle site of drug metabolism in the body, besides chemotherapy drug toxicity research, HepG2 3D cultures have been used to test drug-induced liver toxicity. An example is the investigation on valproic acid (VCA), a widely prescribed antiepileptic drug that has been now used to treat other neurological and psychiatric conditions. Although VPA is usually well tolerated, in rare cases or in an overdose it can cause severe or even fatal liver injuries.

Another application approach for the use of HepG2 cell line is to test the role of natural killer cells in liver injury. Recent studies suggest that natural killer cells have been implicated in inducing hepatocellular damage in patients with chronic hepatitis virus infections. Specifically, the early responses of NK cells against HBV infection can control acute phase of infection and allow development of the adaptive immune system, however failure to control the virus can lead to NK-mediated death of hepatocytes through upregulation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or FASL leading to liver failure. NK cell functions are tightly regulated by a variety of cytokines such as IFN-α, IL-12, IL-15, IL-18, and IL-10 due to their
ability to induce NK activation and polarize them toward degranulation. Increase expression of these cytokines in the liver of in immune activated patients are thus associated with liver injury\textsuperscript{140}.

Furthermore, HepG2 cell in monolayers has been tested previously and the susceptibility to NK cell-mediated killing, was verified\textsuperscript{108}, however studies support that the ability of NK cells to control solid tumors is usually not efficient\textsuperscript{141,142}. For example, attempts to treat solid tumors by natural killer cell immunotherapy have been unsuccessful although in vitro NK cells are effective killers towards cells originating of solid tumors\textsuperscript{143,144}.

\textbf{Future perspective}

Currently, there is limited knowledge related to NK cell infiltration, migration and cytotoxic activity within solid tumors but also how the microenvironment of tumors effects NK cell response. With our system, future investigation could lead to a better knowledge about NK cell properties and about the factors that control and regulate the NK cell response against solid tumors. Furthermore, our method could be beneficial for future studies associated to the activity of NK cells not only against solid tumors, but also for testing their effectiveness against other suspension cells lines. An interesting future study could be the comparison between the heterogeneities against different leukemia cells other than 221 that we have used in this project. Such cells could be for example the K562 cells\textsuperscript{145}.

Little is currently known about the molecular characteristics of the ‘serial killers’ that makes them greatly efficient against tumor cells. Therefore, further investigation of these cells is needed to decode their characteristic cytotoxic behavior, both against suspension tumor cells and solid tumors. A future study could also involve a comparison between the molecular characteristics of inactive, active and serial killer cells to test for example the distribution of inhibitory and activation receptors. This could be achieved through subset clonal expansion and subsequent molecular examination and characterization. This however requires a technological progress to allow collection of the NK cells of interest from individual wells. Especially for the small size wells, this can be quite difficult to accomplish. Another suggestion for future investigation could be to alter NK cell activation. Since we have been only using IL-2 as activating agent, it would be interesting to investigate the potential of other cytokines or cytokine cocktails for NK cell activation.

Furthermore, in the tumor formation system, besides cell lines we could expand towards using cells originated from primary tumors, or even create 3D cultures of different cell types and molecules known to be existing in tumor microenvironments such extracellular matrix proteins. This could be an advantage for testing NK cell activity under more in vivo-like conditions. Our method is not restricted to studies of NK cells or immune cells, but could also
could be adapted to other applications where observation of individual cell–cell interactions is critical.

However, to address these interesting biological challenges either in the immune system field or any other applied biological field, we need to overcome some limitations as described previously related to the device design. A suggestion involves an upgrade of the ultrasonic device by introducing a perfusion system for medium exchange during the experiments. This could be great progress for our system especially for extended time experiments. Besides reduction of the preparation steps, perfusion could also be beneficial to avoid disturbing or abolishing the cells or tumors during medium exchange. Furthermore, an upgrade of the system towards an increased number of wells would definitely be beneficial for acquiring better statistics per individual experiment.
Chapter 5: Conclusions

Growing cells separated from their original tissue under controlled conditions, has been the initial step towards decoding the mechanisms of cellular functions and processes. Most conventional cell-culturing techniques consist of the immersion of large populations of cells in a biocompatible fluid (medium) where measurements result the averaged outcome of individual cell processes. However, due to heterogeneity within cell populations, averaged measurements can be misleading and insufficient to describe unique individual cell characteristics. To date, conventional techniques are gradually abandoned as cell-culture methods, while miniaturized cell culture systems gain territory since they offer several advantages. Mainly they can provide a more in vivo-like cellular environment to ensure more reliable cell-based assays but also they require less sample volumes and general preparation reagents and repetitions\textsuperscript{51}.

In this project, we have developed a multiwell microchip system to facilitate investigation of individual cell characteristics by imaging high numbers of cell-cell interactions at the single cell level. Later we integrated the multiwell microchip to a well-established ultrasonic cell manipulation system that was previously used to trap particles or cells within microfluidic channels\textsuperscript{64,117}. After system biocompatibility was confirmed through several proliferation experiments, we used the multiwell chips to confine cells, either within the physical boundaries (silicon walls) or with ultrasound-induced acoustic boundaries (pressure nodes). Thus, we could establish interactions between NK cells and cancer cells for studying individual NK cell properties such as killing and immune synapse formation.

Our results indicate a heterogeneity within the NK cell population related to their cytolytic response against targets, with a subset to be more prone to kill several surrounding targets in a consecutive manner. In contrary we have observed NK cells incapable to induce target cell lysis although they were maintained in contact for several hours. Further investigation and mostly molecular characterization of these subpopulations could reveal future potential immunotherapy approaches. Nevertheless, in vivo conditions, tumors are usually not composed of single cells but cells are assembled together through tight junctions to form solid tumors. We hypothesized that utilizing the ultrasonic device’s potential to induce cell contacts, if applied to appropriate cell types could lead to formation of individual solid tumors within the wells.
Our results confirmed the theory of solid tumor formation in the multiwell microdevice. However, several unsuccessful attempts verified the significance of high radiation forces in order to increase the number of successful events without using any surface coating or gels. When we applied high voltage ultrasound in a temperature-controlled system, we effectively induced 3D growth of human hepatocellular carcinoma HepG2 cells in parallel. Subsequently, we used the HepG2 tumors as targets to examine the killing and infiltration properties of natural killer cells for up to several days. As expected, we observed a dependency of the tumor outcome and whether the tumor would continue to grow or it would be abolished, on the NK cell number per tumor volume.

From the results presented and discussed in this Thesis, we can conclude that our system can be a useful tool for studying the heterogeneity within cell populations as well as addressing solid tumor related challenges. A suggested future goal of the presented method is to use the solid tumors for optimizing chemotherapy protocols. But also for exploring the limitations of our immune system against tumor growth, and thus investigate the possibilities to increase immune activity.
Chapter 6: Acknowledgments

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Chapter 7: Bibliography


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