Precise cell manipulations and imaging of cellular responses

Methods developed using microfluidic, 3D-printing and microfabrication technologies

NIKOS FATSIS-KAVALOPOULOS
Abstract

It is at the heart of biological and medical research to try and understand how cells communicate with each other, and how cells respond to alterations in their environment, including treatment with different drugs. There is in this context a continued need for better methods that allow researchers to precisely manipulate cells and their microenvironment and to study the resulting responses using high-resolution live microscopy. This thesis presents the development and implementation of several devices that addresses these needs.

A novel microfluidic device called the cell assembly generator (CAGE) was created to generate precisely composed cell clusters of different cell types; the first of its kind. Experimental evidence demonstrated that the CAGE chip can be used to study paracrine signalling in tailor-made cancer cell clusters composed of up to five cells.

A high-throughput microfluidic chip for rapid phenotypic antibiotic susceptibility testing was developed and tested using 21 clinical isolates of Klebsiella pneumoniae, Staphylococcus aureus and Escherichia coli against a panel of antibiotics. Stable minimum inhibitory concentration values were obtained from this system within 2-4 hours with high accuracy to the standard method.

3D-printing was used to create a modular and affordable time-lapse imaging and incubation system, called ATLIS. This system enables researchers to convert simple inverted microscopes into live cell imaging systems, where images and movies of living cells can be recorded using a regular smartphone.

Finally, a strategy was developed for the generation of modular microfluidic systems using 3D-printed moulds for PDMS casting, to enable studies of leukocyte adherence to differentially treated endothelial cell populations in the same field of view and under the same conditions.

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One must try and find the Work they should be doing, find the Story they should be telling and find the Place to start the doing and the telling from
  V.M. Fatsi

There is nothing like looking if you want to find something. You certainly usually find something if you look, but it’s not always quite the something you were after
  J.R.R. Tolkien
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III Rodrigo Hernández Vera, Emil Schwan, Nikos Fatsis-Kavalopoulos, and Johan Kreuger (2016). A Modular and Affordable Time-Lapse Imaging and Incubation System Based on 3D-Printed Parts, a Smartphone, and Off-The-Shelf Electronics PLOS ONE 11(12): e0167583

IV Rodrigo Hernández Vera, Paul O’Callaghan, Nikos Fatsis-Kavalopoulos, and Johan Kreuger (2019). Modular microfluidic systems cast from 3D-printed molds for imaging leukocyte adherence to differentially treated endothelial cultures. Manuscript in revision

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# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Background</td>
<td>11</td>
</tr>
<tr>
<td>BioMEMS : Tools for <em>in vitro</em> studies</td>
<td>14</td>
</tr>
<tr>
<td>BioMEMS development using microfabricated tools and microfluidic principles</td>
<td>14</td>
</tr>
<tr>
<td>Additive Manufacturing – “3D printing” – in BioMEMS development</td>
<td>18</td>
</tr>
<tr>
<td>Interactions between cells and their environment</td>
<td>20</td>
</tr>
<tr>
<td>Controlling the environment during <em>in vitro</em> experiments</td>
<td>20</td>
</tr>
<tr>
<td>Testing the response of bacteria to antibiotics <em>in vitro</em></td>
<td>21</td>
</tr>
<tr>
<td>Interactions between cells</td>
<td>24</td>
</tr>
<tr>
<td>Studying the paracrine interactions that facilitate cancer progression</td>
<td>24</td>
</tr>
<tr>
<td>Studying the interplay between leukocytes and the endothelium of blood vessels as a part of the inflammatory response</td>
<td>25</td>
</tr>
<tr>
<td>Aim of this thesis</td>
<td>28</td>
</tr>
<tr>
<td>Present investigations</td>
<td>29</td>
</tr>
<tr>
<td>Paper I: Formation of precisely composed cancer cell clusters using a cell assembly generator (CAGE) for studying paracrine signaling at single-cell resolution</td>
<td>29</td>
</tr>
<tr>
<td>Conclusions</td>
<td>29</td>
</tr>
<tr>
<td>Paper II: A high-throughput fluidic chip for rapid phenotypic antibiotic susceptibility testing</td>
<td>32</td>
</tr>
<tr>
<td>Conclusions</td>
<td>32</td>
</tr>
<tr>
<td>Paper III: A Modular and Affordable Time-Lapse Imaging and Incubation System (ATLIS) Based on 3D-Printed Parts, a Smartphone, and Off-The-Shelf Electronics</td>
<td>33</td>
</tr>
<tr>
<td>Conclusions</td>
<td>35</td>
</tr>
<tr>
<td>Paper IV: Modular microfluidic systems cast from 3D-printed molds for imaging leukocyte adherence to differentially treated endothelial cultures</td>
<td>36</td>
</tr>
<tr>
<td>Conclusions</td>
<td>37</td>
</tr>
<tr>
<td>Discussion and Future perspectives</td>
<td>40</td>
</tr>
<tr>
<td>CAGE and rAST chip: Papers I and II</td>
<td>40</td>
</tr>
<tr>
<td>ATLIS and the modular microfluidic strategy: Papers III and IV</td>
<td>41</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>BioMEMS</td>
<td>Biological MEMS</td>
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<td>BMD</td>
<td>Broth microdilution</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>FDM</td>
<td>Fused Deposition Modeling</td>
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<td>G-</td>
<td>Gram stain negative</td>
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<tr>
<td>G+</td>
<td>Gram stain positive</td>
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<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>LCD</td>
<td>Liquid crystal display</td>
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<tr>
<td>MEMS</td>
<td>Micro Electro Mechanical Systems</td>
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<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<tr>
<td>P2Y</td>
<td>purinergic G protein-coupled receptor</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol biphosphate</td>
</tr>
<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
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<tr>
<td>rAST</td>
<td>Rapid Antibiotic Susceptibility Testing</td>
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<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SLA</td>
<td>Stereolithography</td>
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<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
Introduction

Background

With the term cell interactions we describe all the ways by which cells communicate or influence one another and their environment. Interactions between cells govern a plethora of biological processes, therefore the study of the ways in which cells communicate and interact stands at the heart of experimental biological research. Even our efforts to look for life when exploring the stars focus in detecting interactions between cells and their environment.

In this work we focus on specific interactions between cells, and between cells and their environment (Figure 1).

In the human body, complex tissues and organs are dependent on the intricate and information-rich interactions between individual cells. Through intercellular communication, mediated by the release and uptake of chemical signals as well as by direct cell-to-cell contacts, complex biological processes are organized.

Interactions between cells and their environment also affect cell behavior. Cell proliferation, differentiation and migration are all shown to be regulated by environmental cues. The loss of cell communication and the failure of cells to interact can even lead to catastrophic effects, such as uncontrollable cell proliferation and disease.

Together with the interactions of cells within the human body, understanding the ways microorganisms and especially bacteria interact is of paramount importance. As antibiotic resistant strains are becoming more common, mortality rates, medical costs and hospitalization times for bacterial infections are constantly growing. Some estimates even state that if left unchecked, in the next 30 years antimicrobial resistance might be responsible for as many as 10 million deaths per year and a loss of 100 trillion USD in production capabilities. Studying how bacteria are affected by antibiotic exposure is important for controlling the rising problem of antibiotic resistance.
The need to study and manipulate cell behavior, both in human cells as well as in microbes, has been a driving force for the development of new methods that study cell interactions in vitro.

New methods that study the interactions between different cell types, as well as cells and their environment have yielded great insight into understanding complex diseases like cancer and diabetes\(^9,10\). Novel approaches on antibiotic testing have led to new insights into microbial resistance\(^11\).

Complex in vitro assays and devices have been created in order for researchers to be able to reproduce interactions between cells. These devices, often referred to as BioMEMS\(^12\) or biological-microelectromechanical systems, are designed to provide researchers with a high level of experimental control over the cell microenvironment. They are often applied to replicate physiological cell conditions that involve multiple cells and stimuli. BioMEMS (Figure 2) usually have multiple micrometer-scale, mechanical and electrical parts to effectively manipulate microscopic biological samples (such as cells) and to miniaturize biological assays\(^12\).
In order for any BioMEMS device to both support living biological samples and expose them to different conditions, handling of microscopic volumes of liquid is needed. The implementation of microfluidics is therefore commonly used to be able to precisely regulate the cell microenvironments and imitate in vivo conditions.

Although many published micro-devices have the potential to increase the quality of research aimed at understanding interactions between cells, the wider scientific community has yet to fully adopt them. The fabrication of BioMEMS is a complex process that requires specialized training and precision-engineered equipment. Multistep microfabrication is most often needed for the construction of a microfluidic device, which is a labor intensive and expensive process. The high complexity in the fabrication procedures of BioMEMS is a limiting factor for the development of new assays, as it only permits specialized and well-funded research groups to develop customized devices.
The recent developments in additive manufacturing, commonly known as 3D printing, have started to dramatically simplify the fabrication of micro-devices. High-precision desktop 3D printers are now becoming available to the public. These printers are capable of creating structures with a precision down to 5 micrometers (μm), offering an affordable and simpler substitute for traditional microfabrication methods that require work in a clean room facility. Thus, with the increased ease of use and reduced expenses of fabrication methods of additive manufacturing, one no longer requires a high level of expertise in microfabrication to be able to make microdevices. As 3D printing becomes more accessible, so does the ability for researchers to create custom assays for the study of complex biological processes.

By using the techniques and advantages of BioMEMS the work presented here was focused on developing novel methods that allow for accurate and controllable study of cell behavior in vitro.

BioMEMS: Tools for in vitro studies

BioMEMS development using microfabricated tools and microfluidic principles

Every modern-day electronic device is composed of integrated circuits and micrometer-scale electronic components. The technologies and methods developed in the 1950s to fabricate the first integrated circuits are the precursor to what we know as microfabrication today.

Soon after the initial development of microfabrication methods for integrated circuits, techniques were developed to create microstructures on silica wafers. That is how the first Micro Electro Mechanical Systems (MEMS) came into being. With the development of liquid handling microdevices the first BioMEMS were developed. Since then, devices created by microfabrication methods have been used for the development and miniaturization of biological assays and devices. A commonly used technique in the field of biological micro-devices is a multistep process known as lithography (Figure 3). The first part of this process, known as photolithography, involves the use of photoresists. A photoresist is a chemical that polymerizes when exposed to light of specific wavelength. For the work presented here, SU8 photoresist was used. Initially, during the photolithography process, the surface of a silica wafer is coated with photoresist. The coated wafer is placed under a light source underneath an opaque photomask. The photomask restricts light, so light only reaches the sensitive photoresist where the desired final structures should appear on the wafer. Where the photoresist is exposed to light through the mask, it crosslinks. Non-crosslinked photoresist is stripped from the wafers to reveal the fully hardened structures where the
wafer was exposed to light. These structures can then be used as a mold for the fabrication of the final device in the second part of the process.

Figure 3. Illustration of the steps of the SU8 lithography microfabrication process: a wafer is first coated with SU8 and then a photomask is applied on it. Following light exposure through the mask the exposed photoresist crosslinks. During chemical stripping, only crosslinked photoresist remains on the wafer forming the desired geometry.

The second part of photolithography is called replica molding. It involves the use of polydimethylsiloxane (PDMS). PDMS is a biocompatible, air permeable, two-part silicone that is used widely as a material for devices in biological applications. The previously constructed wafer is used as a mold to cast PDMS (Figure 4). First, the PDMS prepolymer and polymerization agent are mixed, initiating a curing process that can be accelerated by heat. The mixture is poured on the surface of the wafer mold, and left to cure in an oven. After curing, the PDMS cast is peeled from the wafer mold. The imprint features the various channels and mechanisms that form the final device. Usually, if that device is to be used for imaging by microscopy, the feature side of the PDMS cast is bonded to microscope glass to allow for optical clarity when imaging.
Figure 4. Illustration of the replica molding process: PDMS is poured on the wafer featuring the structures fabricated with photolithography, and left to polymerize. After curing, the resulting cured cast of PSMS has an imprint of the wafer structure comprising the desired device.

The combination of microfabrication techniques with the biocompatibility and versatility of PDMS has given rise to a myriad of Bio-chips. PDMS being inherently inert, transparent and easily bonded to glass allows for precise manufactured assays suitable for high-resolution microscopy and live imaging (Figure 5). Taking advantage of the gas permeability of PDMS, these devices are suitable for stably culturing and sustaining cells for long periods of time under very controlled experimental conditions.

Figure 5. Cells in culture in a microfluidic chip for several hours cell proliferation on chip (as shown on paper III)

Most cells used in cell interaction studies require a liquid environment to survive. Therefore, it is imperative for chips that deal with live-cell studies to handle and control liquids. Due to the small size of most chips, liquid handling is done in microscale volumes and with very slow fluid flow rates.
The part of fluid mechanics that deals with the control of microscale volumes and flow of liquids is called microfluidics. In fluid mechanics, the Reynolds number is a ratiometric, dimensionless measurement used to predict fluid behavior. Flows with high Reynolds numbers are generally turbulent, whereas flows with low ones tend to be more laminar. Microfluidic flows usually exhibit low Reynolds numbers, far less than 1 ($Re \ll 1$). In these flows, referred to as Stokes or creeping flows, advective inertial forces are much smaller than viscous forces*. With viscosity rather than inertia, being the driving property in microflows they exhibit some very different characteristics, that contrast to macroscale fluid mechanics. As is presented in this work, laminar flows can be developed with no mixing between two adjacent streams of liquid (Figure 6). That creates an environment where diffusion is the only driving force carrying substances from one stream to another. That allows for the precise creation of reagent gradients in microfluidic devices. Exposing cells to precise gradients of chemical cues is also an important tool in in vitro studies (as discussed on paper I, II and IV).

* Mathematical representation of the effect of low $Re$ numbers on fluid equations is found in Supplementary Figure 1.
idic chips to use flow to apply mechanical stimuli such as shear stress, even complex in vivo environments, like the lining of a blood vessel (as described in paper IV) can be modeled using microfluidic approaches.

Additive Manufacturing – “3D printing” – in BioMEMS development

Additive manufacturing, also known as 3D printing, is the process of making a component with raw material that is deposited or polymerized in the desired shape in a step-by-step manner. This differs from traditional manufacturing techniques, where material often is subtracted from a stock shape. Modern 3D printers are easy to use, affordable and have a high level of manufacturing precision. Regarding the development of BioMEMS, 3D printers provide designers with a less labor-intensive alternative to the complex process of photolithography. Instead of having to apply and re-apply the steps of a photolithographic protocol in order to make complex 3D structures, designs are fabricated automatically with little human intervention.

Creating an object with 3D printing has 3 main steps: design, printing and post processing. Design involves the complete modeling of the final part in computer software. All desired features that make a part functional can be drawn with precision and then reproduced in the actual part. The time it takes to go from design to finished part is usually very small in 3D printing, which makes the technology a very efficient and reliable tool for prototyping.26

Printing will manufacture the design into a physical object. Many technologies of 3D printing exist,27 and two printing processes are of specific interest to this thesis (Figure 7): Fused deposition modeling (FDM) printing and stereolithography (SLA) printing. In FDM printing, filaments of different materials are fed into an extruder that precisely heats them just above their glass transition temperature. This now malleable material is deposited one layer at a time through a thin nozzle. This process is most commonly used to make large parts with no particular requirements for physical or material properties.28

SLA printing is a technology attributed to be the new generation of lithography techniques29. Thin layers of photcurable resin are exposed to light and polymerized, usually by a light beam (laser spot SLA printing) or a light pattern (LCD SLA printing), which reflects the design of the part. Parts manufactured with SLA printing are usually of higher precision than those made with FDM printing and can exhibit specific properties, such as being impermeable to water, which makes this method ideal for the creation of assays for biomedical applications and handling of liquids30.
Figure 7. Breakdown of the additive manufacturing techniques used in this work

Post-processing is the part of the process that takes place after the actual printing. Through finishing, parts can acquire additional properties such as chemically treated edges\(^{30}\), and activated or inactivated surfaces\(^ {30,31}\).

Both FDM and SLA printing have found great use in biological applications. Especially in the manufacturing of precision biomedical equipment that is expensive to and difficult to fabricate\(^ {32}\).

An alternative additive manufacturing technique not featured extensively in this work but with great promise in life science research is Bio printing. Although 3D printing of biological materials (extracellular matrix with or without cells) is still in its initial stages of development, custom printers have been created that can be used for tissue engineering\(^ {33-37}\) and custom design of prosthetics\(^ {38-40}\). Applications both in research and clinical settings already exist\(^ {41,42}\) that demonstrate the successful application of this technology in a variety of assays. Hydrogels and various extracellular matrix scaffolds are already currently being bioprinted\(^ {43}\), both to aid in vitro experiments and to facilitate regeneration and wound healing. 3D-printed tissue grafts have been successfully transplanted in live joints and ligaments\(^ {44}\), skin, bones and vasculature\(^ {45}\). The ultimate goal of 3D printing biological materials would be to print live tissue and organs based on computer models or real life scans\(^ {45}\).

All the developing techniques of additive manufacturing have greatly impacted the field of microdevices. It is now possible, with 3D printers, to manufacture bioengineering equipment, in-house and with great precision. This has made 3D printing an ideal tool to disseminate microdevices and to streamline the fabrication of biomedical assays\(^ {46}\).
Interactions between cells and their environment

Controlling the environment during *in vitro* experiments

Living cells both affect and are affected by their environment. They are constantly probing their surroundings and responding to changes. Surrounding temperature, chemical composition, mechanical properties and availability of resources such as oxygen, temperature, pH and nutrients are important environmental parameters that affect cellular behavior (*Figure 8*).

![Figure 8. Environmental factors and their interplay with the cell](image)

Cells extracted from human tissue operate normally when they are at 37°C. When the temperature deviates from this physiological level, even by a few degrees, cells experience thermal stress. Such stress leads to significant changes in cell transcriptome expression and their expected response to stimulation. Therefore, in order to keep results consistent and reliable, it is important to maintain a steady and controlled temperature while performing biological experiments.

Cells normally reside in environments with a specific composition of mechanical stimulation and physical properties. Cells like chondrocytes and hematopoietic stem cells (bone marrow cells) require a very confined environment with a tightly knit extracellular matrix (ECM) and constant mechanical stimulation. On the other hand, certain leukocytes normally flow in the bloodstream or migrate into tissue, and therefore need to be unconstrained in suspension. Even the fate of stem cells is partially determined by the physical characteristics of their environment. Replicating the physical
conditions of the native environment of different cell types is an important challenge to all in vitro studies.

In the liquid environment of most cells, the osmotic and hydrostatic pressure of the cell medium impacts multiple signaling pathways and alters cellular responses to stimulation\textsuperscript{54-56}. Cancer cells inside tumors may experience a high pressure environment, up to 40 times the hydrostatic pressure of healthy tissue\textsuperscript{57,58}. Abnormally high tumor pressure has been demonstrated to affect tumor invasiveness, angiogenesis and metastasis, as well as on the efficacy of chemical treatments\textsuperscript{59}. Therefore, increasing the hydrostatic pressure in vitro to match the interior environment of a tumor is an important tool in assays that aim to model cancer cell behavior\textsuperscript{55,56}. The chemical properties of the cell suspension medium are also of great importance. Medium pH, oxygen levels and growth factor concentrations all affect cell gene expression.

Controlling the chemical composition of a cell’s surroundings is also important in perturbing cell behavior. Chemical or growth factor stimulation of cells has been a well-established technique in experimental biology. Impeding tumor cells’ ability to cope with low pH values has been shown to have therapeutic effects\textsuperscript{60}. Studies of hypoxic and hyperoxic conditions as well as oxidative stress have shown that extremes in oxygen concentration contribute to the development of cardiovascular disease, inflammation and even diabetes\textsuperscript{61,62}.

All aforementioned studies were performed by tightly controlling the chemical composition of the cell environment, illustrating the necessary function environmental control plays in in vitro studies of cell behavior (as discussed on papers I-IV). The advancements in 3D printing and the field of BioMEMS has allowed for the development of precise and affordable devices that can be used to closely monitor, maintain and modulate the cell environment, as shown in papers I-IV of this work.

Testing the response of bacteria to antibiotics in vitro

Even before the establishment of laboratory sciences, the effect certain substances have in treating infections was well-documented. Examples of antimicrobial treatments exist through the ancient world in Egypt, China and Greece\textsuperscript{63}. In 1928, Sir Alexander Fleming discovered penicillin, a discovery that lead to the development of purification methods and large scale production of antibiotics as an effective medical treatment of bacterial infections.

Antibiotics used in medicinal treatments act by inhibiting microbial growth or destroying microorganisms while having minimum impact on the cells and tissue of the patient\textsuperscript{64}. Unfortunately, though, any one antibiotic does not present a permanent, long-term solution, to bacterial infections.

The constantly shifting genetic landscape of microbiomes makes the bacterial genome extremely responsive to any environmental conditions\textsuperscript{65}. Bact-
certain bacteria have high population numbers and short generation times. So in an environment with a strong antibiotic selection pressure any naturally occurring resistant mutants are very strongly selected for and eventually take over. Natural selection, also plays a key role in the spread of resistant strains; as antibiotics are applied and eradicate sensitive organisms, resistant ones are left behind to reproduce and spread. Furthermore, resistance does not have to be a product of the spread of a beneficial genetic mutation, as it can also be acquired through horizontal genetic transfer.

Horizontal genetic transfer in bacteria can happen both directly, from cell-to-cell contact, or indirectly, through the cell environment. Bacterial cells are able to take up and incorporate genetic material directly from their environment in a process known as transformation. Bacterial DNA can also be transported between cells through cell-to-cell contact (bacterial conjugation) or introduced through a virus (transduction). All these mechanisms accelerate the spread antibiotic resistance, even among different species of bacteria.

A clear solution to the rising problem of resistance is the development of new antibiotics. For several decades, antibiotic research has aimed to develop new antibiotics faster than microbial strains developed resistance to treatment. Unfortunately, in the last three decades, the rate of discovery of new antibiotics has greatly been reduced, while the rate of resistance has risen (Figure 9).

![Figure 9](image)

Figure 9. The decline in antibiotic discovery (adapted from). Starting from the 1960s discoveries of new antibiotic families has been decreasing and becoming more sporadic, leading to a discovery void of virtually no new antibiotic families in the past 30 years.

The lack of discovery of new antibiotics together with antibiotic overuse has led to the rise of antibiotic resistant strains worldwide (Figure 10). As a different avenue of approaching the global resistance problem, antimicrobial research is now exploring new methods to adapt and refine antibiotic treatment regimes. Two factors have proven most important for both patient
care and reducing antibiotic resistance\textsuperscript{73,74}: selecting the correct antibiotic to treat an infection and treating with the correct concentration.

Knowing which antibiotics a strain is more susceptible to will allow clinicians to design highly efficient treatment regimes\textsuperscript{75}. Important factors to the success of a treatment is also the concentration of antibiotics it applies. Too high a concentration may have toxic off-target effects on patient health\textsuperscript{76,77}. Even more, treating with high concentrations needlessly increases exposure to antibiotics in the environment and drives the buildup of resistance further. Too low a concentration, on the other hand, would not be effective in eradicating the infection completely\textsuperscript{78,79}. It would apply a selection pressure to the established bacterial population for the more resistant organisms, eventually giving rise to a resistant strain\textsuperscript{80}. A useful metric to address these issues is the Minimum Inhibitory Concentration (MIC). A MIC value for a specific antibiotic is a characteristic of a strain and it is the minimum concentration of that antibiotic that has inhibitory effects in that strain’s growth.

![Figure 10. Resistant isolate proportion for Klebsiella pneumoniae strains in Europe in 2018. A clear rise of resistant strains can be observed. Image courtesy of the ECDC surveillance ATLAS](image)

The need to explore the susceptibility of a strain prior to treatment, in a fast and precise way, is a driving force behind the development of a multitude of new BioMEMS.

The ability of BioMEMS to quickly and precisely control the conditions of a cell culture is optimal for studying effects of antibiotics in bacterial cultures \textit{in vitro}. Isolating smaller quantities of bacteria and exposing them to treatments yields more precise results in less time than traditional macroscopic approaches\textsuperscript{81}. 


\[ \text{Figure 10. Resistant isolate proportion for Klebsiella pneumoniae strains in Europe in 2018. A clear rise of resistant strains can be observed. Image courtesy of the ECDC surveillance ATLAS} \]
The application of BioMEMS in the study of antibiotic resistance has given rise to the field of rapid antibiotic susceptibility testing (rAST). In rAST bacterial samples are tested for their response in antibiotics faster than traditional assays, allowing for refinement of treatment, resulting in conservative and effective use of antibiotics (as discussed on paper II).

Interactions between cells
Studying the paracrine interactions that facilitate cancer progression

The human body has been estimated to be composed of more than 600 different cell types and around $10^{14}$ individual cells. All these different cells are in a constant state of sending and receiving chemical signals, governing the interactions necessary for a multicellular organism to function properly. Tumors do not exist in isolation from the rest of the body, as they are often surrounded by multitudes of different physiological cell types and are exposed to a variety of chemical signals. The chemical communication between tumor cells and other host physiological cell types is of particular interest to this work. Through paracrine signaling, the tumor microenvironment both affects and is affected by cells from surrounding cell groups.

For example, paracrine ATP stimulation has been shown to influence ion transport in cancer cells (also discussed on paper I). Chemokines released from mesenchymal stem cells have been shown to increase colon cancer cell migration and proliferation. A paracrine loop between macrophages and breast cancer cells has been shown to increase metastatic behavior. Adipokines secreted from adipose tissue have been shown to impact tumor growth through a paracrine pathway. Paracrine stimulation has also been correlated even with an increase of tumor invasion.

Conversely, tumor cells can use paracrine stimulation to affect normal cells. Angiogenesis and vascularization has been shown to increase as a result of paracrine stimulation with vascular endothelial growth factor secreted from primary tumors. Immunosuppression has been observed as a result of factors secreted by glioblastoma cells. Even chemoresistance has been noted in colon cancer as a result of the tumor secreting inflammatory cytokines and drug metabolizing enzymes.

Studies in paracrine signaling are regularly performed in vivo and animal models are most often used. They are most suited to mimic human disease and provide researchers with a complex, comprehensive representation of a studied phenomena. However, most animal models are sensitive to interspecies differences and their results are do not always replicate biological and physiological mechanisms of the human body. Furthermore, maintain-
ing animal models is an expensive and laborious task, accountable to ethical considerations.

In vitro studies, on the other hand, are less complex and best suited to isolate and study specific interactions. Human cells of different cell types can be isolated and combined in various in vitro tissue and microtissue models. With these reductionistic, in vitro, cell interaction models, the interplay between specific cell types can be studied, isolated from the interference of other cells. The chemical environment the cells are exposed to can be precisely manipulated, regulated and monitored (as discussed on paper I). The biochemical interaction between the secreting and receiving cells of a paracrine response can be investigated more clearly. Finally, in vitro models tend to be more replicable compared to in vivo ones, allowing for more consistent experimental outcomes.

With the use of microfluidic chips, manipulations even down to the level of single cells can be accomplished. Cells can be combined and rearranged on chip to form any constellation necessary for paracrine studies in a very precise way (as discussed on paper I). Microfluidic devices are also capable of manipulating very small volumes of reagents, making them ideal for exposing cells or cell aggregates to different chemical cues with high precision.

Studying the interplay between leukocytes and the endothelium of blood vessels as a part of the inflammatory response

The blood that flows in the circulatory system, is a non-homogenous biological fluid. Suspended in water, a mixture of several different cell types, growth factors, electrolytes, ions, proteins, dissolved gasses and nutrients make up the blood stream. The circulatory system that is responsible for dispersing and circulating blood is composed of a mesh of blood vessels.

Blood vessels and their surrounding tissue are dynamic environments where substances and cells are exchanged constantly. Blood vessels occur in a variety of sizes; from capillary vessels between 5 to 10 μm wide, to veins and arteries that can be as wide as 2 cm.

The cells that line the interior surface of blood vessels are called endothelial cells and they form the interface between all that circulates within the blood and other tissues. Acting as a barrier, the endothelium is also responsible for gating intravasation and extravasation of circulating cells to and from the blood stream.

During the inflammatory response, the endothelial cells play a key role in the recruitment of circulating leukocytes from the blood stream. Inflamed tissue produces inflammatory cytokines that initiate a signaling cascade leading to the endothelium being activated.

The activated endothelial cells start expressing selectins, a cell adhesion protein family that recognize specific polysaccharides on the surface of the
leukocytes. A circulating leukocyte first comes into contact with the blood vessel wall and a tethering between the selectins and the oligosaccharides on the surface of the leukocyte is formed. The leukocyte still experiences forces from the blood flow that antagonize the tethering of the selectin-mediated adhesion, which begins with low affinity. Consequently, the leukocyte begins to roll on the endothelial wall in a capture and release interplay between the selectin adhesions and the force of the blood flow (Figure 11). The balance between the blood flow forces and the tethering of the selectin adhesion is broken once the leukocyte activates its integrins. The activated integrins bind to proteins on the surface of the endothelium with high affinity and stably hold the leukocyte in place, halting the rolling and allowing the leukocyte to attach to the endothelial wall. Once fully attached, the leukocyte starts transmigrating through the blood vessel into the inflamed tissue guided by chemoattractant gradients.

Figure 11. Illustration of a blood vessel with the attachment states of leukocytes: A leukocyte is presented as circulating: the leukocyte is carried by the bloodflow; captured: the leukocyte is tethered by the selectins (blue) expressed by the endothelial cells but not fixed in place. Its integrins remain unactivated (red); rolling: the leukocyte is rolling on the endothelium in a catch and release interplay between the selectin interaction and the force of the blood flow; attached: the leukocyte has activated its integrins and remains fixed in a position on the endothelium (green); adherent: the leukocyte is adherent to the endothelial layer.

The interaction between endothelial cells and leukocytes is a crucial part of the inflammatory response. It is an integral part of understanding the mechanisms of disease and infection as well as in studying tumor spread and metastasis. The complex dynamics of two different populations of cells under shear stress and flow, actively adhering to each other, are not trivially replicated in vitro. The process of rolling and attaching is heavily impacted by the blood flow. Macroscopic assays that provide solutions for simply coculturing leukocytes with endothelial cells do not model effectively the full scope of the interaction.
To successfully create an environment where biologically relevant flow can be applied to multiple populations of cells, microfluidic chips can be used\textsuperscript{115–117}. Multiple BioMEMS exist that model the geometry of blood vessels\textsuperscript{118,119}, while sustaining a culture of endothelial cells. Using microfluidic technology, flow rates corresponding to the situation in small blood vessels can be achieved and used to study effects of shear stress on leukocyte adhesion\textsuperscript{120}. Using microfluidic assays,\textsuperscript{121} the entire leukocyte adhesion cascade, from capture to transmigration, can be studied \textit{in vitro}. The use of BioMEMS has also enabled controlled testing of the effects of different inflammatory cytokines on the leukocyte adhesion\textsuperscript{122} (also discussed on paper IV).
Aim of this thesis

The aim of this work was to combine traditional and newly developed design and fabrication techniques to create novel methods in the study of cell interactions. More specifically the aims of the papers in this thesis were:

**Paper I** To develop a novel system for precise assembly of multicellular heterogeneous cell clusters, in order to study paracrine interactions between breast cancer cells and pancreatic cells.

**Paper II** To create a multiplex microfluidic system capable of exposing bacterial colonies in gradients of antibiotic concentration in order to perform rapid antibiotic susceptibility testing.

**Paper III** To present a customized and inexpensive solution based on additive manufacturing and off-the-shelf electronics to increase the accessibility of time-lapse imaging techniques.

**Paper IV** To create a new modular microfluidic platform using additive manufacturing, in order to study endothelial and white blood cell interactions with integrated experimental controls.
Present investigations

Paper I: Formation of precisely composed cancer cell clusters using a cell assembly generator (CAGE) for studying paracrine signaling at single-cell resolution

This paper describes a novel microfluidic device called Cell Assembly Generator (CAGE) (Figure 12A), which is capable of generating heterogeneous cell clusters of predefined cell number and cell type. The CAGE chip implements three consecutive functions to produce the eventual cell cluster: single cell trapping/isolation, cell ejection and cell clustering (Figure 12B). It was made using replica molding on photolithographically produced molds.

Firstly, a cell population in suspension is allowed to flow through the loading channel of the system. An array of hydrodynamic single cell traps allows single cells to be isolated from the general population. Once all traps are populated with single cells, the system can switch flow direction and eject the cells from the traps via a cross flow, which directs them into the clustering chambers. When the cells have been moved into the clustering chambers, the traps are empty and can accept new rounds of trapping and ejecting. By repeating the trapping and ejecting protocol multiple times, multicellular clusters are created one cell at a time (Figure 12C). Different cell populations can be used in each trapping step to generate heterogeneous cell clusters of specific proportions with a single cell resolution (Figure 12D).
Figure 12. A) The CAGE chip: A scheme outlining the microfluidic chip. B) The clustering mechanism consisting of a single cell trap and clustering chamber. C) Cluster generation by consecutive trapping and ejecting single cells. D) Generation of multicellular heterogeneous clusters, two cells of each of the two cell types was used. The two populations are stained green and red to illustrate the population origin of the heterogeneous clusters.
CAGE was implemented in an experiment to investigate interactions between breast cancer and pancreatic cells. Some breast cancer cells are known to migrate and establish secondary tumors in different organs, such as the pancreas. To explore this scenario, we used CAGE to generate defined clusters of pancreatic beta cells and breast cancer tumor cells. In these cell clusters, we studied calcium responses in the cancer cells evoked by paracrine adenosine triphosphate (ATP) secretion from pancreatic β-cells.

We designed a model cluster where a single ATP secreting pancreatic β-cell was clustered together with 3 ATP sensitive (receptor expressing) breast cancer tumor cells. Once the cells were clustered in the cluster chamber, a potassium solution was introduced into the device to depolarize the excitable pancreatic β-cells to secrete ATP. The potassium stimulation was shown to have no effect on the cancer cells.

Pancreatic β-cell-derived ATP binds to P2Y receptors of cells activates the conversion of PIP₂ to IP₃. IP₃ in turn stimulates the release of Ca²⁺ from the ER into the cytosol, (Figure 13A). Ca²⁺ fluctuations in all cells were monitored over time using a fluorescent calcium reporter and high-resolution confocal microscopy. Clustered cells in a well-defined space allowed for visualization of individual cells, allowing precise analysis of the spatial relationship between the cell and its response to stimulation. A clear correlation was discovered between the distance of a cancer cell to the pancreatic cell and its accumulated Ca²⁺ change. The greatest response was observed from cancer cells in closest proximity to a β-cell (Figure 13B).

Our imaging protocol takes place after the initial stimulation with ATP and subsequent release of Ca²⁺ from the ER to the cytosol. Given the timing of the measurements, we considered, that the observed differences in Ca²⁺ concentration in the cytosol of the cancer cells were mainly a measure of Ca²⁺ clearance back in the ER. Therefore we set out to investigate how the mechanism of Ca²⁺ clearance back into the ER affects the cells’ response to ATP stimulation (Figure 13B). To achieve this, we designed a different type of cell cluster consisting of: one pancreatic cell, two cancer cells and two cancer cells treated with an ER SERCA pump inhibitor. This treatment disables the cells’ ability to clear Ca²⁺ from the cytosol back into their ER. Having no ability to replenish the Ca²⁺ in their ER, led to the eventual depletion of the ER stores of Ca²⁺ in the treated cells. By comparing the response of the SERCA disabled cells of the clusters to untreated cells, we observed that the depletion of ER Ca²⁺ due to the cells’ inability to clear Ca²⁺ back in their ER rendered the cells insensitive to ATP stimulation (Figure 13B).

† Data shown on Paper I supplementary Figure 2C
Conclusions

In this paper, we developed a novel microfluidic device. CAGE is a microfluidic chip capable of generating precise multicellular clusters of exact composition. Once assembled, cell clusters can be cultured and treated on chip and imaged with high resolution confocal microscopy. The benefit of having a device that can precisely generate reductionist tissue models was illustrated by its implementation in a study of paracrine signaling effects on cancer cells. With the use of the device, single ATP source cells were clustered together with multiple cancer cells. The clusters generated were isolated from peripheral cells and external stimulation, so that only the stimulation by a single cell was analyzed. This analysis showed that a spatial relationship exists between the position of the cancer cells, in relation to the ATP secreting cell, and their Ca\textsuperscript{2+} levels over time. Cancer cells in closest proximity to the ATP-source revealed the greatest accumulated change in Ca\textsuperscript{2+} levels.

Figure 13. The experimental for paracrine signaling. A) Illustration of the stimulation and subsequent paracrine signaling. B) Distant dependent Ca\textsuperscript{2+} fluctuations of cancer cells (left) and SERCA pump inhibited cancer cells (right) exposed to the ATP secretion of a single beta cell.
Minimum Inhibitory Concentration (MIC) is a value that describes the minimum concentration of an antibiotic to inhibit microbial growth. MIC is traditionally determined in rAST (rapid antibiotic susceptibility testing), by exposing bacteria populations to antibiotics using distinct concentration steps (serial dilutions). MIC is thus determined as an extrapolation between two discrete concentration values, not by performing a direct measurement on a continuous spectrum\textsuperscript{124}. Methods that do use continuous concentration gradients (like disk diffusion assays), however, can be very time consuming and produce low accuracy results\textsuperscript{124}.

In the present study we introduce a high-throughput rAST chip. This device performs highly accurate susceptibility tests using a continuous spectrum of antibiotic concentrations. The device consists of eight microfluidic test chambers in which bacteria are seeded in suspension in an agarose gel. A microfluidic circuit is used to flow antibiotic solutions around the test chambers. Every chamber is flanked by two fluidic channels. On one side an antibiotic dissolved in culture medium flows, acting as an antibiotic source channel. On the other, plain culture medium flows, acting as a concentration sink (Figure 14A). This source-sink layout allows antibiotics diffuse into the agar of the test chambers to form a continuous active gradient of antibiotic concentrations. The gradient spans the entire range of the test chambers and is actively maintained by constant flow of the two channels. MIC values are determined by quantifying bacterial growth in the different parts of the chamber, that correspond to different antibiotic concentrations along the antibiotic gradient (Figure 14B).

To measure bacterial growth on chip and to determine MIC for every chamber a custom image analysis algorithm was implemented. The algorithm takes into account both bacterial growth and changes of culture behavior over time to determine MIC values.

To operate the microfluidic chip, 3D printed components were designed that assemble the complete system (Figure 14C). The 3D printed components contribute to the system’s operation so that eight possible antibiotics can be tested on the same time, multiplexing measurements.
Having developed all the necessary components of the high-throughput rAST system, we benchmarked its performance by testing 21 clinically relevant bacterial isolates and six antibiotics. MIC measurements were obtained on average under three and a half hours for all antibiotics (as low as two and a half hours for some). To gauge the assay’s accuracy, all MIC values obtained by this system were compared with ones obtained with the laboratory standard broth microdilution method (BMD) (Figure 15).

82% of all G- and 67% of all G+ strains had essential agreement with the standard MIC values (measurement within a two-fold dilution step of the reference value) (Figure 15).

Categorical agreement of this systems measurements was also high with 85.6% of strains in total categorical agreement (classified correctly as susceptible, intermediate or resistant strain).
Figure 15. Essential agreement of MICs calculated with high-throughput rAST chip: Comparison of MIC values obtained through this system with standard reference MIC values obtained with BMD.

Conclusions

As shown by its benchmarks, this high-throughput rAST system has provided for a robust and accurate solution, which could be the foundation for true clinical rAST. With this method at its heart, an instrument is currently under development by Gradientech AB that will deliver clinical rAST capabilities.
Paper III: A Modular and Affordable Time-Lapse Imaging and Incubation System (ATLIS) Based on 3D-Printed Parts, a Smartphone, and Off-The-Shelf Electronics

To be able to study cell interactions, one needs to be able to observe and monitor them reliably. In modern experimental biology, this is often achieved by acquiring images from a microscope in set intervals of time, while constantly controlling the conditions of the cell culture environment. Acquiring all the devices necessary and implementing them in biological experiments raises the financial cost to levels that may be unattainable by many research groups.

This paper describes ATLIS, an Affordable Time-Lapse and Imaging System that is an accessible alternative to common time lapse equipment. The ATLIS system is composed of four individual subsystems (Figure 16): an image acquisition module, a temperature control module, a light control module and a humidity control module. Every subsystem can operate independently from the rest on the same control unit, allowing researchers to pick and choose only the modules required by their specific application. The ATLIS system is made using 3D printed parts, easily obtainable electronics and a smartphone. The image acquisition system uses a smartphone and a custom fixture that allows for any type of smartphone to be affixed to the ocular of most common microscopes. Through custom developed software, images can be acquired in set time intervals.

Inexpensive environmental control was achieved by using custom incubation chambers designed to be small and application specific. These miniaturized chambers are heated by an active airflow generated by small inexpensive heating units (Figure 16). The light control module consists of a motorized shutter that interrupts the light path to the stage. According to the image acquisition needs, the shutter can open to expose the cells to light during image acquisition. The control system to operate the temperature regulator and the microscope shutter was designed based on an Arduino. A PID controller was implemented to regulate the temperature.

Combining the function of all these subsystems, cells were cultured and imaged (Figure 17). All the modules were designed to be compatible with assays based on imaging of cells in microfluidic chips, where evaporation can be a key problem. To address this, a humidity module was designed. When connected to the temperature control, the humidity module can maintain a suitably humid environment, while maintaining a highly controllable temperature profile. Cells cultured in microfluidic devices in the system were shown to migrate and proliferate.

36
Conclusions

ATLIS presents a modular and affordable alternative to time-lapse and incubation systems. 3D printing technologies, together with custom and highly controllable subsystems, were used in the design of the system. This approach reduces system complexity and price, enabling researchers access to important time-lapse capabilities for the studies of cell interactions.
Paper IV: Modular microfluidic systems cast from 3D-printed molds for imaging leukocyte adherence to differentially treated endothelial cultures.

Microfluidic devices can be hard to manufacture and difficult to use, preventing many researchers from implementing such assays in their work. This study describes a novel strategy that uses 3D printing to create easy-to-make, modular microfluidic systems. With the use of 3D printed molds, we were able to create multifunctional microfluidic chips on demand. Furthermore, we generated 3D printed alignment parts that allow multiple microfluidic chips to be applied one after the other on the same substrate. The versatility of this modular approach was demonstrated by creating an assay for simultaneous studies of interactions of a leukocyte cell population with two different, distinct endothelial cell populations (Figure 18).

During the inflammation response, immune cells selectively bind to the luminal surface of endothelial cells in order to transmigrate into surrounding inflamed tissues. To model these conditions, two chip modules were used to facilitate tethering of leukocytes onto endothelial populations. First, a chip was used that enables the co-culture of two populations of endothelial cells in close proximity to each other. Then a second chip was applied that allows for fluid flow of leukocyte cells across the top of this cell culture (Figure 18).

Figure 18. The modules of the system: The barrier module was used to seed two cell populations in close proximity. The barrier module was thereafter replaced with a flow module using an alignment tool, and leukocyte interaction experiments carried out.

First, the barrier module was vacuum bonded to a glass slide. Endothelial cells were seeded and allowed to attach overnight to the barrier module (Figure 18). The design of the barrier module allows two different treatments to be applied side-by-side. This study used a cytokine TNFα treatment to one of the endothelial cell populations (simulating an inflammation response) while the other population was kept untreated as a control. After the
end of this treatment, the barrier module was released from the glass and replaced by the flow module. A population of leukocytes was allowed to flow across the two endothelial populations. The close proximity of the two endothelial populations (representing inflamed and normal endothelia) allowed simultaneous imaging (Figure 19).

Figure 19. Leukocyte rolling assay: leukocyte rolling and adherence to activated endothelial cells. Leukocytes (green) were passed over the two endothelial populations. The TNFα-treated endothelial cells retained more leukocytes compared to the control.

Conclusions
This modular microfluidic strategy is a new approach to microfluidic device development, which focused on accessibility of equipment and modularity. This method was developed to produce microfluidic chips using standard 3D printers. This study shows how modular chips implemented one after the other are able to perform complex assays, much like traditional lab-on-a-chip approaches, but without the need for complicated, integrated functions. This modular system enabled a clear, quantitative analysis of leukocyte adhesion on endothelial cells.
Discussion and Future perspectives

CAGE and rAST chip: Papers I and II

Papers I and II represent attempts to create novel laboratory research tools. They aim to expand a researcher’s abilities to manipulate cells and form specific environmental conditions.

In papers I and II the respective microfluidic chips are used in the study of specific biological problems - paracrine signaling in cancer and antibiotic susceptibility testing - but the mechanism that both systems offer can be applied to study other phenomena as well. CAGE’s ability to isolate single cells and combine them in specific constellations can be applied to a variety of models that require co-culture of multiple cell types. The rAST chip’s ability to expose bacterial populations to controlled gradients of substances can be an important tool in the study of bacterial population dynamics. Although the systems presented here were meant to address specific limitations in existing experimental procedures, their cell manipulation capabilities will allow broader use in their respective fields of research.

The key functionality of each device is represented in a core element: in the case of CAGE the clustering element; and in the rAST chip the test chamber. Both assays are designed in such a way that multiple core elements can be combined in a single microfluidic chip. In this way, creating high throughput systems that potentially allow for high number of biological replicates. In order to perform live biological experiments, the chips are equipped with capabilities to culture living cells. Culture medium is perfused in both systems and they are shown to be capable of sustaining live cell cultures. Different treatments to the cells are also necessary for experimentation and both devices have the ability to stimulate cells with different solutions.

Both assays are designed for optical imaging of cell interactions (fluorescence in CAGE and QuicMIC visible bacteria growth in rAST). Although compatibility with high resolution microscopy is an important criterion for BioMEMS, downstream analysis of the biological samples is also a desirable feature. The systems in their current stage of development only provide optical, end-point measurements. Future development can expand this capacity to include methods that would allow for harvesting of the biological samples, and further biochemical analysis.
ATLIS and the modular microfluidic strategy: Papers III and IV

The successful implementation of new BioMEMS in experimental laboratories relies on two important factors: access to high-end experimental equipment and expertise in the manufacturing and handling of microfluidic chips. Those two requirements are an obstacle for many labs to adopt new experimental procedures and technologies.

Papers III and IV represent new approaches in the way we produce and handle BioMEMS. This constitutes a concerted effort to reduce the previously mentioned mitigating factors and to facilitate the spread of BioMEMS technology. To achieve this, both approaches of Papers III and IV were developed to be modular, inexpensive and accessible.

Modularity imbues the systems with versatility and room to grow. In the case of ATLIS, functions performed by the system are divided between individual subsystems that can operate independently or combined. The versions of the system can thus be tailor-made to meet specific needs. In the barrier module with flow, complex microfluidic manipulations are broken down into distinct steps implemented by separate devices. Complex microfluidic chips can be substituted with multiple simpler ones that collectively perform the same functions.

Expanding on the existing technologies is also made easier because of our modular approach. ATLIS subsystems work independently, allowing the development and incorporation of modules that control different environmental parameters into the system with minimum effort. Currently, a fluorescent imaging and filtration module is being developed that would give ATLIS systems the ability to perform fluorescent imaging. This method’s library of chips can also be expanded to include different microfluidic functions. A gradient generating chip has already been developed and can be incorporated into potential applications. With all chips following the reversible bonding and alignment methods a wide toolbox of microfluidic functions can be created for researchers to pick and choose from.

In addition to modularity, affordability is also an important factor in the spread of new technologies. ATLIS is an environmental control and imaging system made from relatively inexpensive 3D printed parts and consumer electronics. This makes it a viable option for labs with tight budgets and a good alternative for groups that wish to reduce bottlenecks that arise from shared equipment and facilities. The modular microfluidic strategy of paper IV represents an alternative way to manufacture and produce microfluidic components using 3D printing. Microfabrication was previously accessible only through expensive equipment and cleanroom facilities, but now can be attained by non-expert personnel in common laboratory settings.
Together, papers III and IV represent an appealing approach for future directions, applying rapid prototyping technology to create affordable and accessible alternatives to complex systems currently in use.
Cell interactions are the focal point of multiple disciplines and fields. The study of how cells interact with each other and/or their environment encompasses a great variety of biological and chemical processes, physical properties and mechanical actuations. Most modes of interaction are complex, exhibiting multiple steps and intricate interdependent subsystems. To address that, reductionist in vitro models are of great use. In vitro, specific phenomena can be isolated and studied separately, without having to account for the overwhelming level of in vivo complexity.

The need for detailed in vitro models has given rise to Biological Micro Electro Mechanical Systems (BioMEMS). Microscale devices and machinery that have found use in generating detailed, highly controlled models used in most experimental biology disciplines. The use of MEMS and their peripheral biomedical equipment has the capacity to increase availability of assays to the scientific community, provide researchers with a deeper level of experimental detail, generate a higher level of experimental control and reduce time to complete complex, time-dependent assays. These advantages of BioMEMS were demonstrated in the papers that constitute this work.

In paper III, new approaches in fabrication were used to design ATLIS, a system that controls parameters of the cell environment. With this system, the study of cell interactions is available to a wider scientific community.

It is that drive of all experimentalists to have access to new level of control on their assays, motivating paper I. In the development of CAGE, traditional microfabrication approaches were used to develop a complex cell interaction study system. CAGE creates reductionist and precise cell clusters that act as templates to study cell behavior.

The same need to simplify complex in vitro models was the subject of paper IV. The microfluidic strategy presented an alternative approach in microfluidics, combining 3D fabrication and modular microfluidics to simplify what is usually a complex in vitro system.

The combination of new and traditional approaches in BioMEMS is also what allowed for the development of a new system to study the interactions of bacteria with antibiotics, featured in paper II. The high-throughput rAST chip, presented uses traditional dark field microscopy coupled with innovative gradient forming chambers to perform antibiotic susceptibility testing rapidly and reliably.
In this work I sought out to create new methods in the study of cell interactions, not by simply implementing traditional BIOMEMS capabilities on new problems, but by combining experimental biology, additive manufacturing and microfluidic design. The results of this effort are systems that incorporate all the advantages of BioMEMS with the wide availability of 3D printing. Systems designed, developed and manufactured were applied to deconvolute complex biological problems, and enhance *in vitro* assays with better accuracy, control and precision.
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‘Never laugh at live dragons’126
Målet med detta arbete har varit att utveckla nya tekniker för att manipulera celler och avbilda deras respons på olika behandlingar, med syftet att tillhandahålla verktyg för studier av cellinteraktioner. Verktygen som presenteras i denna avhandling tillverkades med nya tekniker inom mikrofabricering och utnyttjar de speciella fysikaliska fenomen som förekommer i små vätskevolymer, så kallad mikrofluidik.

Till att börja med presenterar vi två nya metoder för att studera cellinteraktioner; CAGE, ett verktyg som möjliggör studier av hur cancerceller kommunicerar med celler de inte är i direkta kontakt med, samt rAST chip, ett system som snabbt kvantifierar effekten av antibiotika mot patogena bakterier. Vidare presenteras två nya tillvägagångssätt för hur vi kan utveckla metoder för att studera cellinteraktioner; ATLIS, ett ekonomiskt och modulärt mikroskopsystem samt, en modulmetod för att i laboratoriet studera interaktioner mellan immunceller och celler i blodkärlen.

CAGE tillåter forskare att hantera mikroskopiska cellgrupper med ett specifikt antal celler eller celltyper, med möjligheten att exponera dessa cellgrupper för olika behandlingar och mäta hur cellerna påverkas i realtid. Med denna metod lyckades vi visa att avståndet mellan cancerceller och celler i exciterbara β-celler är avgörande för kalciumregleringen.


ATLIS är ett system tillverkat av olika 3D-printade komponenter som tillåter att man kan hålla celler i en naturlig, kontrollerad miljö under tiden de utsätts för experiment under ett mikroskop. Till skillnad från existerande system kan detta tillverkas direkt av enskilda forskare, och ger möjlighet för biomedicinsk sjukspetsforskning också i resurssvaga miljöer. ATLIS kärna bygger på automatisk avbildning i mikroskopet med hjälp av en vanlig smartphone tillsammans med en specialskriven applikation. Vi utvärderade systemets prestanda genom att mäta cellförökning under olika experimentella förhållanden.
Περίληψη με απλούς όρους στα Ελληνικά

Η συγκεκριμένη διδακτορική διατριβή αποτελεί μια προσπάθεια προς την ανάπτυξη νέων μεθόδων για την χειραγώγηση κυττάρων και την απεικόνιση κυτταρικών συμπεριφορών. Σκοπεύει στο να παράσχει νέα εργαλεία στην μελέτη κυτταρικών αλληλεπιδράσεων. Οι συσκευές και τα εργαλεία που παρουσιάζονται εδώ έχουν κατασκευαστεί με καινοτόμες μικροκατασκευαστικές μεθόδους και λειτουργούν εκμεταλλεύομενες τις φυσικές αρχές που διέπουν την κίνηση ρεύστων μικροσκοπικών όγκων.

Αρχικά παρουσιάζουμε δύο νέες μεθόδους για την μελέτη κυτταρικών αλληλεπιδράσεων. Το CAGE: μια συσκευή που μελετά τον τρόπο επικοινωνίας των καρκινικών κυττάρων, με κύτταρα με τα οποία δεν βρίσκονται σε άμεση επαφή και το rAST chip: ένα σύστημα που επιτρέπει τον έλεγχο της δραστικότητας των αντιβιοτικών εναντίον συγκεκριμένων βακτηρίων.

Έπειτα παρουσιάζουμε δύο νέες προσεγγίσεις στον τρόπο με τον οποίο αναπτύσσουμε μεθόδους για την μελέτη κυτταρικών αλληλεπιδράσεων. Το ATLIS ενα προσιτό εναλλακτικό σύστημα μικροσκοπίας, και μια αρθρωτή προσέγγιση στην εργαστηριακή μελέτη των αλληλεπιδράσεων που έχουν τα κύτταρα του ανοσοποιητικού συστήματος με τα κύτταρα των αιμοφόρων αγγείων.

Το CAGE επιτρέπει στους επιστήμονες να φτιάξουν μικροσκοπικές ομάδες κυττάρων, από συγκεκριμένο αριθμό και είδος κυττάρων. Επιπλέον, έχει τη δυνατότητα να εκθέτει αυτές τις ομάδες σε διαφορετικές ουσίες και να ελέγχει πώς επηρεάζονται τα κύτταρα. Χρησιμοποιώντας το CAGE καταφέραμε να αποδείξουμε ότι η απόσταση ενός καρκινικού κυττάρου από ένα κύτταρο του πάγκρεα, είναι σημαντική για το τρόπο με τον οποίο το καρκινικό κύτταρο προσδιορίζει τα επίπεδα του ασβεστίου του.

Το rAST chip είναι μια συσκευή που εκθέτει 8 δείγματα βακτηρίων ταυτόχρονα σε συγκεκριμένα αντιβιοτικά. Έπειτα με την βοήθεια υπολογιστικής ανάλυσης εικόνας το σύστημα προσδιορίζει την αποτελεσματικότητα των αντιβιοτικών πάνω στα δείγματα. Με βάση αυτό τον υπολογισμό μπορεί να μετρηθεί η αποτελεσματικότητα πιθανών μοντέλων θεραπείας με αντιβιοτικά. Πιστοποιήσαμε την αποτελεσματικότητα του συστήματος χρησιμοποιώντας κλινικά δείγματα βακτηρίων. Δείχνουμε ότι με χρήση του συστήματος η μέτρηση της αποτελεσματικότητας των αντιβιοτικών είναι ακριβής και πολύ πιο γρήγορη από προγενέστερες μεθόδους ευρείας χρήσης.
Το ATLIS είναι ένα σύστημα φτιαγμένο από στοιχεία που προέκυψαν με τρισδιάστατη εκτύπωση. Επιτρέπει την καλλιέργεια κυττάρων στην διάρκεια εκτέλεσης πειραμάτων στο μικροσκόπιο. Αποτελείται από έναν αρθρωτό σχεδιασμό, του οποίου το κάθε στοιχείο συνεισφέρει στην διατήρηση των περιβαλλοντικών συνθηκών μιας κυτταρικής καλλιέργειας. Το σύστημα συμπληρώνεται από την αυτόματη ανάκτηση εικόνων των κυττάρων στο μικροσκόπιο με τη χρήση ενός κινητού τηλεφώνου και ενός ειδικού προγράμματος ανάκτησης φωτογραφιών. Αξιολογήσαμε την λειτουργία του συστήματος, απαθανατίζοντας με αξιόπιστο τρόπο κυτταρικο διπλασιασμό σε διάφορες πειραματικές διατάξεις.

Τέλος αναπτύχθηκε μια νέα προσέγγιση στην μελέτη των αλληλεπιδράσεων ανοσοποιητικών κυττάρων και κυττάρων των αιμοφόρων αγγείων. Χρησιμοποιώντας τεχνικές τρισδιάστατης εκτύπωσης, αναπτύξαμε νέες μεθόδους στην κατασκευή μικροσυσκευών και δημιουργήσαμε μια νέα μέθοδο για την διαδοχική τους χρήση. Χρησιμοποιώντας αυτή τη διαδοχική προσέγγιση, δημιουργήσαμε 2 συσκευές που δρούν επικοινωνιακά η μία στην άλλη. Η πρώτη δημιουργεί το περιβάλλον ενός αιμοφόρου αγγείου και η δεύτερη εκθέτει ανοσοποιητικά κύτταρα σε αυτό. Αυτή η προσέγγιση αξιολογήθηκε με βάση παγιωμένες πειραματικές μεθόδους και αποδείχθηκε να έχει την ίδια πειραματική ισχύ.
References


T. Boland, T. Xu, B. Damon and X. Cui, Biotechnology journal, 2006, 1, 910–917.


C. L. Ventola, Pharmacy and Therapeutics, 2014, 39, 704.


68 R. K. Holmes and M. G. Jobling, in *Medical Microbiology*, ed. S. Baron, University of Texas Medical Branch at Galveston, Galveston (TX), 4th edn., 1996.
75 R. DerSimonian and N. Laird, *Controlled Clinical Trials*, 1986, 7, 177–188.


Supplementary Information

Supplementary Figure 1

\[
Re \frac{Du^*}{Dt^*} = -\nabla^* p^* + (\nabla^*)^2 \overrightarrow{u^*} + \left[ \frac{\rho g x_o}{\mu u_0} \right] \overrightarrow{l_g}
\]

Inertia

viscosity

pressure gradient

external forces

\[
Re \ll 1
\]

\[
0 = -\nabla^* p^* + (\nabla^*)^2 \overrightarrow{u^*} + \left[ \frac{\rho g x_o}{\mu u_0} \right] \overrightarrow{l_g}
\]

ratio of gravitational to viscous effects

\textit{S.Figure 1.} Dimensionless Navier-Stokes governing equation of fluid mechanics solved for incompressible flow and Newtonian fluids. The explanation of every term is below in red. In a very low Re environment the effects of inertia in the terms are negated and pressure and viscosity remain as the governing terms of the flow. Depending on the ratio of gravitational forces to viscous effects, the gravitational forces acting on the liquid might also be negligible.
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