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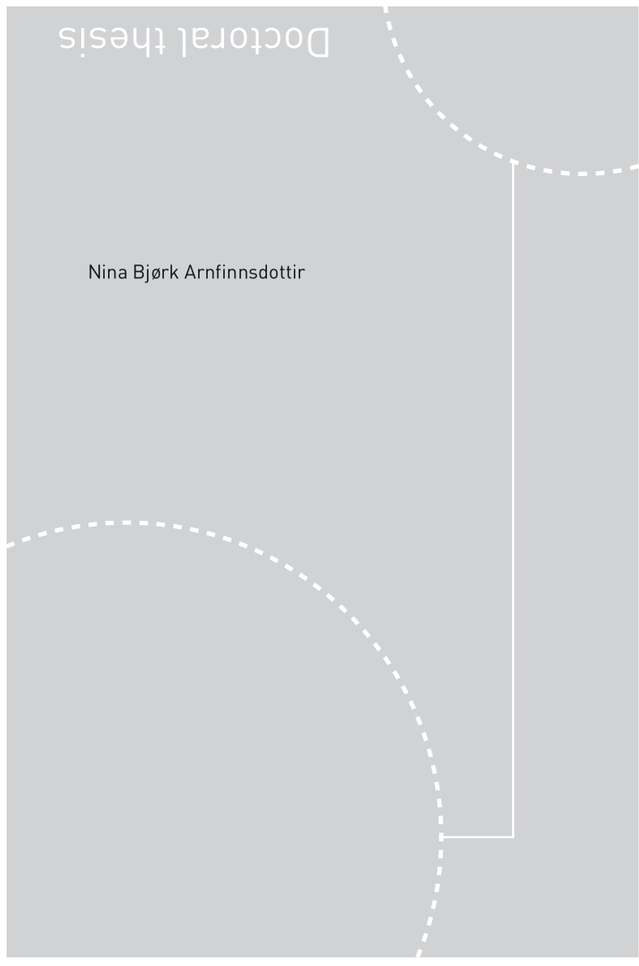
Nina Bjørk Arnfinnsdottir

Microscale tools for the development of bacterial microarrays



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Thesis for the Degree of Philosophiae Doctor

Trondheim, March 2016

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Abstract

Heterogeneity within bacterial populations is a phenomenon that has gained much interest over the last decades. It has been shown that even isogeneous colonies under homogeneous conditions can display different phenotypes. This heterogenous gene expression in bacteria is considered to be an evolutionary developed trait that increases the chance of survival under changing environmental conditions. This also impacts human health as some phenotypic traits can enable bacteria to survive antibiotic treatment, resulting in reoccurring bacterial infections. There is understandably much interest in uncovering the underlying mechanisms of such phenotypic differences, both for optimized medical treatments and to further our understanding of the behavior of bacterial populations.

Standard methods utilized in microbiology are however based on average measurements, and thereby inherently masking the existence of small subpopulations and other rare events. With the emergence of techniques capable of large scale single cell measurements, e.g. flow cytometry, much focus has been put on the understanding of heterogeneity of bacterial populations. There is however a need for single cell measurements that provide time resolution in order to study the dynamics of such phenomena. Such time resolution can be obtained through time laps imaging of bacteria. Large scale single cell measurements could however benefit from an ordered attachment of bacteria onto a substrate

In this thesis I present methods for fabrication of bacterial microarrays, focusing on utilizing methods and chemicals that are applicable in standard biological laboratories. The presented method is based on micro contact printed patterns of chemicals on glass substrates for the selective adhesion of bacteria. Ordered arrays of *Pseudomonas putida* KT2440 were obtained on patterns of polydopamine on glass surfaces coated with a polymer consisting of a poly-l-lysine with grafted polyethylene glycol. Such arrays were utilized to inspect the heterogeneity in expression of green fluorescent protein from two different plasmids carried by the bacteria. The results were comparable to results obtained based on measurements of the same system preformed on a flow cytometer.

The surface paterning technique presented was also adapted for the selective adhesion of alginate microgels onto glass substrates. Encapsulation of cells in such alginate microgels allowed for inspection of three dimensional culture growth and the possibility of selective removal of single microgels utilizing a micropipette controlled by a micromanipulator.

Aknowledgements

There are many that deserve my thanks for helping and supporting me throughout my work with this thesis, so I will keep each mention short. This does in no way reflect the degree of gratitude feel towards each and everyone for their contribution to my time as a PhD student at NTNU.

I would like to thank my supervisor Marit Sletmoen for constructive conversations, positive encouragement and an enthusiastic attitude towards my project. I would also like to thank my co-supervisor Rahmi Lale for opening up the door for me to the exciting world of bacteria and biotechnology.

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I would like to thank my friends Sara, Ingrid, Erna, Christine and Frida for reminding me that there is a life outside of the walls of NTNU and keeping me in shape by being delightful training buddies.

Thank you all!

List of papers

The following papers are included in this thesis:

I **The design of simple bacterial microarrays. Development towards immobilizing single living bacteria on predefined micro-sized spots on patterned surfaces.**
Nina B. Arnfinnsdottir, Vegar Ottesen, Rahmi Lale and Marit Sletmoen.
Plos One, 2015, 10.
The study was designed by NBA and MS. The experiments were performed by NBA and VO, and NBA wrote the paper in collaboration with MS. All authors contributed to the revision and discussion of the paper.

II **Heterogeneity in GFP expression in isogenic populations of P. putida KT2440 investigated using flow cytometry and bacterial microarrays**
Nina B. Arnfinnsdottir, Astrid Bjørkøy, Rahmi Lale and Marit Sletmoen.
RSC Advances, 2015, submitted.
The study was designed by NBA, MS and RL. All experiments were performed by NBA. AB wrote the script used by NBA and MS for subsequent data analysis. The paper was written by MS and NBA. All authors contributed to the revision and discussion of the paper.

III **Microarrays of alginate gel beads for analysis of encapsulated microorganisms**
Nina B. Arnfinnsdottir, Armend G. Håti, Gianluca Etienne, Bjørn T. Stokke, Esther Amstad and Marit Sletmoen.
Article in preparation.
The study was designed by NBA and AGH. All experiments were performed by NBA and AGH. GE synthesized surfactant used in the microgel production and EA contributed with design options for microfluidic devices. The article is written by NBA, AGH, BTS and MS. All authors contributed to the revision and discussion of the paper.

Related publications
The following papers fall outside the scope of this thesis:

1. **The size and shape of three water-soluble, non-ionic polysaccharides produced by lactic acid bacteria: A comparative study**

Marianne Ø. Dalheim, Nina B. Arnfinnsdottir, Göran Widmalm and Björn E. Christensen.
Carbohydrate Polymers, 2015, submitted.

Contents

1 Introduction

Cell to cell variability, including its underlying cellular mechanisms as well as its consequences have in recent years become a central topic within life sciences. The interest for this phenomenon is in part motivated by the observed phenotypic heterogeneity of bacterial populations, a heterogeneity which may have a significant effect on the overall behavior of the population[1]. However, most traditional biophysical/biochemical approaches record measurements over large numbers of cells and provide time and population averages. These average values mainly represent the states of highest probability within the population, and information reflecting the inherent population heterogeneity is inaccessible when using these approaches . This insight has motivated a quest for experimental approaches that enable studies of bacterial populations at the single cell level. The overall scope of this thesis has been to develop an experimental approach that makes imaging of high numbers of well separated single cells possible in a non-labor-intensive way.

1.1 Aim of thesis

This thesis focuses on the fabrication of bacterial microarrays. As part of this thesis a novel method for the fabrication of such microarrays is proposed and evaluated. The proposed method is based on micro contact printing used to immobilize single bacteria on micron sized spots on chemically patterned surfaces. More specific claims include:

- Obtain microarrays of live bacteria with single cell resolution.
- Identify a method for microarray fabrication that is fast and applicable in a standard molecular biology laboratory.
- Confirm the efficiency and reliability of the obtained microarrays in studies of bacterial populations through a comparison with excisting techniques.

- Apply the proposed surface functionalization approach to obtain arrays of cell-loaded microgels.

2 Background

2.1 Bacterial heterogeneity

The term bacterial heterogeneity can refer to any variability or diversity that occurs between individual bacteria in a colony. The term thus covers both genetic and phenotypic differences, and is used to describe differences that can be either discrete or continuous. Genetic variation is defined as the diversity in DNA sequences and is brought about by random mutaion, while phenotypic differences are related to differences in which genes are expressed in an organism. The existence of genetic mutations within a bacterial population is expected, and the evolution of the population in response to a changing environment relies on such hereditary differences. The importance and benefit of phenotypic variability is on the other hand not as self evident. Distinct phenotypic differences can be observed even in isogenic populations under homogeneous conditions and this phenotypic variation has been shown to be important for bacterial survival and adaptation to changing environments[2–5].

One example of phenotypic heterogeneity in bacterial populations that is of importance for human health is the existence of persister cells. Persister cells constitute a very small subpopulation of bacteria, and are defined by their ability to survive antibiotic treatment without being resistant to the antibiotic. Their lack of antibiotic resistance becomes clear when, after antibiotic treatment, the few surviving persister cells are regrown to a new colony. A subsequent antibiotic treatment of this new colony renders the same result as the first treatment; close to all the bacteria are killed[5–7].

Persister cells are thought to be an important player in reoccurring bacterial infections. In most cases any lingering persister cells will be taken care of by the immune system after antibiotic treatments. This is, however, not always the case. Infections of tuberculosis can hide in the body for years, and the ability of the bacteria to hide from the immune system has been coupled to persistence[8]. Persistence also presents a

problem in patients with a weakened immune system, as the patient might not be able to fight off the lingering persister cells. Resent research has also coupled the persisters to the difficulty of treating infections caused by biofilms, e.g. on catheters and other implants[9]. It has been suggested that the persisters are protected from immune cells by the exopolymer matrix produced by the biofilm, even when the majority of the cells in the biofilm are killed. This allows the persisters to survive antibiotic treatment and subsequently hide from the immune system as they regrow the biofilm once the treatment is finished[7].

The exact mechanisms behind the persistence is not known, but there is evidence that the persistence is coupled to a transient dormant state where growth is arrested[10] and biosynthetic pathways are downregulated[7]. Resistant bacteria carries a mutation that prevents a drug to bind to its target, and thereby hinders the drug from killing the bacterium. This is in contrast to persistence, where it seems the bacterium shuts down its activity so that when a drug attaches to its target motif, it will be unable to disturb the function of the target. In this way the cell survives, at the cost of its ability to continue dividing.

Heterogeneity in gene expression in bacteria is not limited to persistance. A range of phenomena are coupled to phenotypic variability in bacterial populations. One such phenomenon is the existence of bi-stable populations where cells enter into one of two possible phenotypes[3;4;11]. Among these are the lactose utilization system in *E. coli*, where an all-or-nothing switching behavior between an ON and and OFF state related to the bacterias ability to utilize lactose can be observed depending on the availability of lactose in the environment. The bacteria *B. subtilis* has several such systems, including competence, sporulation or switching between swimming or chaining behaviour in solution[11].

The usefulness of heterogeneity in gene expression can be illustrated by the arabinose utilization system in *E. coli* at low arabinose concentrations. In general, gene

expression in bacteria is often coupled to environmental ques, often related to which nutrients are avaiable. *E.coli* can for instance utilize a range of saccharides as a source of energy, but each saccaharide requires separate systems both for transport through the membrane and enzymes needed to degrade the saccharides. Simultaneously expressing the enzymes needed for all these different pathways costs the bacteria more energy than it produces. The bacteria therefore have developed genetic switches that can turn on or off utilization systems in reaction to the availability of different saccharides[12]. Since gene expression is linked to environmental ques such as local concentration of saccharide, heterogene environments can lead to heterogene gene expression as the bacteria adapts to its current micro-environment. Perhaps more surprising is the observed heterogeneity between different bacterial cells living under identical conditions. Gene expression is, however, controlled by molecules of relative low abundance[13;14], leading to stochastic variations of the availability of such molecules in each bacteria. For single cells this can give rise to a random switching between different phenotypes. For *E. coli*, the arabinose utilization system is turned off unless the bacteria senses arabinose in its environment. At high arabinose consentrations, the utilization system is turned on in all bacteria. The heterogenetic gene expression comes into play when the bacteria are exposed to relatively low arabinose concentrations. At such low concentrations a large heterogeneity in the time period it takes for an individual bacteria to turn on the arabinose utilization system after being exposed to arabinose can be observed (figure 1)[15;16]. The degree of heterogeneity is also concentration dependent. For each bacteria this on/off switching is a result of stochastic events as a result of stochastic noise in the gene expression.

By allowing stochastic noise to play a role in gene expression at low arabinose levels, a sub population of bacteria will turn on the arabinose utilization system very shortly after arabinose becomes available in the environment, while others wait longer. By preventing the simultaneous switching on of the arabinose utilization system in all bacteria, the colony ensures that a subpopulation of bacteria can utilize the small amounts of

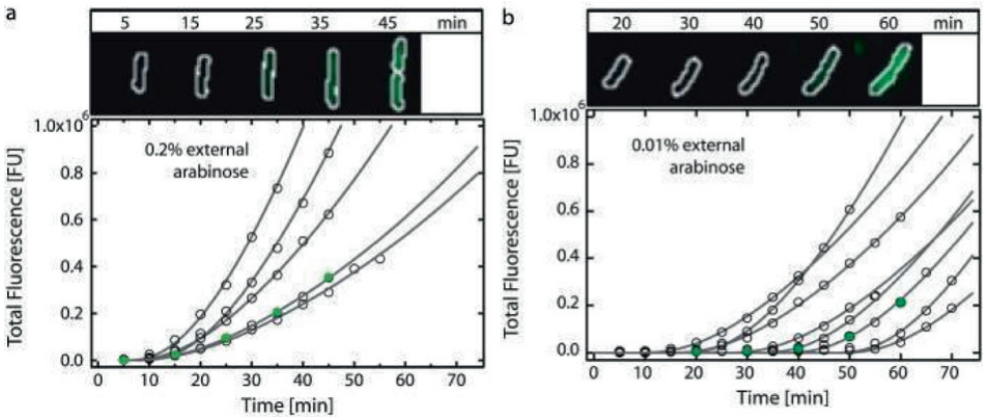

Figure 1: Time laps fluorescent imaging was performed on several single *E. coli* cells induced with 0.2% (a) and 0.01% (b) arabinose to study induction kinetics of the arabinose utilization system at low arabinose concentrations. The turning on of the arabinose utilization system was coupled to fluorescence signals. The induction kinetics of single cell induction could be followed and is displayed with open circles in the plots. The green circles correspond to the bacteria in the image panels. The time from addition of arabinose to the turning on of the arabinose system was highly heterogeneous between individual bacteria, as is evident from the time vs fluorescence curves. Figure reproduced from[15] with permission.

arabinose available in the environment while the others save the energy-cost of turning the system on.

In a larger evolutionary perspective, allowing stochastic noise to affect gene expression and thereby giving rise to several phenotypes even in homogeneous environments can be seen as a bet-hedging strategy for the bacterial colony[13;17;18]. By allowing for small subpopulations of bacteria with less than ideal phenotypes, the colony increases its survival chances when the environmental conditions change[2;13]. Since the different subpopulations are of the same genotype, the genome is preserved if the environment changes and favors these rare traits. This also means that once the environment returns to a favorable state, the colony will reappear exactly as it was. This would not be possible if the adaption was based on genetic mutations. Another useful trait of phenotypic differences arising in colonies is division of labor[17]. Phenotypic heterogeneity allows for

the division of the colony into separate phenotypes dedicated to processes that are either impossible or ineffective for a single cell to perform simultaneously. One such example is the nitrogen fixation and photosynthesis in cyano bacteria. Phenotypic heterogeneity is also an elegant tool for the bacteria to ensure that tasks that are detrimental for single bacteria, but important for the colony, will be carried out by some of the cells in the colony. Even if the task involves the lysis of single bacteria to release nutrients to neighboring cells under nutrient limiting conditions, the genome is still preserved, and new self-sacrificing cells will continue to arise within the colony.

Statistical models have been utilized to desdcribe when and how phenotypic differences can give a evolutionary advantage to a bacterial colony[2]. These models must however be complemented by large scale single cell measurements in order for the phenomena to be completely understood. Some possible techniques are discussed below.

2.2 Measuring heterogeneity

The existence of variability within bacterial colonies has been known since the first half of the 20th century. In the 1940s two papers where published that both describes bacterial variability; Bigger introduced the concept of persister cells[6] and Withell concluded, after a thorough mathematical analysis of survival curves of bacteria after disinfection, that "the different rates of destruction of bacteria is determined essentially by differences in the manner in which the resistance of the organisms are distributed" [19].

Many of the standard techniques commonly applied to the study of bacteria relies on average measurements, thereby inherently masking the existence of small subpopulations of cells with different phenotypes. And even when population based experiments like the ones performed by Bigger and Withell hints of the existence of inter-colony variations, the study of the distribution of different cell phenotypes was, for a long time, impossible due to a lack of technology capable of performing large scale single cell experiments.

This has, however, changed with the emergence of techniques like flow cytometry

and single-cell microscopy in combination with genetically encoded fluorescent reporter proteins.

2.2.1 Coupling fluorescence to gene expression

Phenotypic variations in bacteria are not necessarily visible as differences in physical appearance such as size or shape. In order to study variations in the gene expression there is therefore a need for labeling techniques that can relate the expression of genes to a signal that can easily be measured. This is typically done by employing fluorescent labeling. Fluorescent molecules are defined by their ability to be excited by light of a certain wavelength and subsequently emitting light of a longer wavelength, facilitating the imaging of only the light emitted from the fluorophore through the use of filters or dichromatic mirrors in optical systems.

Gene expression involves the transcription of DNA to RNA and the subsequent translation of RNA into proteins. Quantifying RNA or protein products present in a cell therefore reflects gene expression rates. There are many strategies employed in order to tag inter-cellular proteins with fluorescent molecules[20], but for studies of gene expression genetic labeling of the products of the gene of interest is preferred.

Genetic labeling refers to the insertion of genes that produces fluorescent proteins into a genome. These fluorescent proteins can be expressed on their own or in fusion with other proteins. Genetic labeling of gene expression products became possible with the discovery and cloning of the green fluorescent protein (GFP)[21;22]. After the discovery of GFP, a whole family of fluorescent proteins with different excitation and emission spectra have been developed[22].

When utilizing genetically labeled fluorescence signals, once the gene is expressed the cell typically produces enough of the fluorescent molecules for the cell to display a uniform intensity. These signals can be detected by both flow cytometers and fluorescence microscopes, and the overall intensity of the fluorescent signal can be correlated to

the concentration of fluorescent molecules. Fluorescence microscopy can in addition be utilize to follow temporal dynamics and localization of fluorescent proteins within single cells[23].

2.2.2 Flow cytometry

Flow cytometers quantify properties of single cells, one at a time, as they are passed through a beam of laser light(figure 2). The control of the passage of cells in front of the laser is achieved by hydrodynamic flow focusing, and up to several thousands of cells per second can be detected. Detectors are placed around the point where laser hits the liquid stream, and both side- and forward-scattered light and fluorescent signals can be detected. The forward scattered signal is related to cell size, whereas the side scattered signal can be related to intra-cellular granulation[24]. Fluorescent signals from the cells are dependent on what fluorescent markers are utilized, and can report on a vast variation of properties. Modern flow cytometers offers the simultaneous detection of up to 11 different fluorescent markers. The high throughput and single cell resolution offered by flow cytometers makes them ideal for the detection of distributions of properties as well as the existence of small subpopulations within a larger colony. This makes flow cytometry an important tool for gaining insight into phenomena related to heterogeneity. Flow cytometry measurements have for instance been utilized to study the regulation of noise in the expression of a single gene in *B. subtilis*[26]. Similarly, noisy gene expression in eukaryotic cells has been explored by flow cytometry[27] and the noisy expression of proteins in yeast cells have been shown to have some structure related to the function of the proteins[28].

Fluorescence based detection in flow cytometry can also be coupled to cell sorting mechanisms. This is typically achieved by electrostatic sorting where the jet containing cells is broken into droplets and the droplets carrying cells of interest are given a certain charge. The charged drops are deflected to specific containers and the sorted cells can

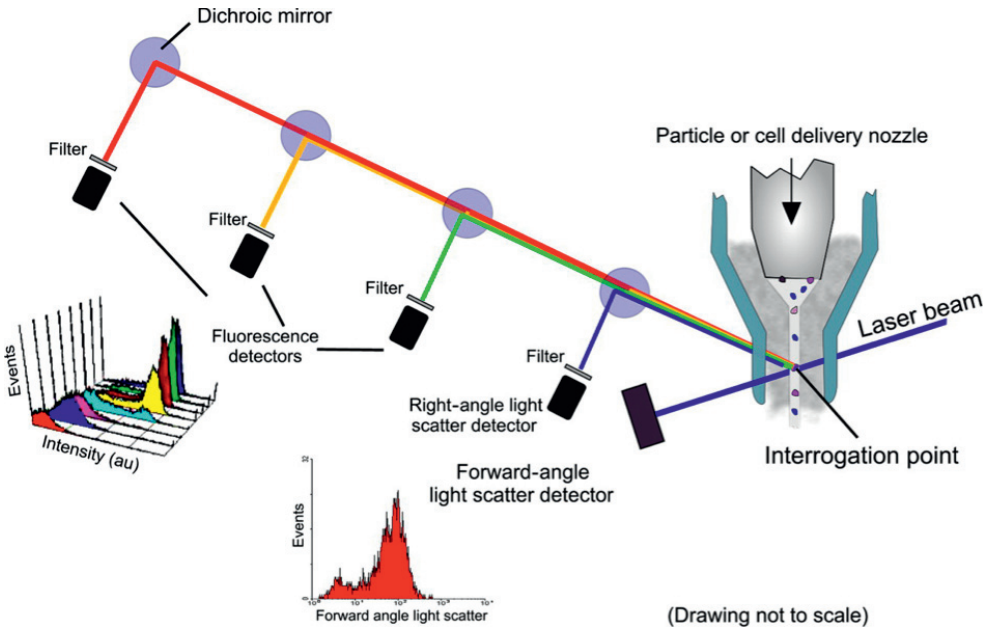

Figure 2: Illustration of the main components of a flow cytometer: particles or cells are passed by a laser beam one by one in a liquid stream. Single cell resolution is achieved by hydrodynamic focusing of the liquid. As the cells passes the laser beam scattered light and fluorescent signals are detected allowing for detection of multiple parameters. Figure reproduced from[25] with permission.

be collected for further studies[25]. Such cell sorting has for instance been utilized to isolate persister cells whose gene expression was subsequently compared to normal cells using a mRNA microarray[29]. Once sorted and collected, cells of special interest can be investigated further utilizing techniques such as microscopy, cultivation or proteomics-based technologies[25;30–33].

2.2.3 Single cell microscopy

When it comes to measurements of phenotypic heterogeneity the perhaps largest drawback of the flow cytometer is the lack of temporal resolution of the single cell measurements. Once a cell is detected and measured it can not be tracked and followed over

time. Fluorescence microscopy allows for the detection of the same parameters as a flow cytometer (size, shape and fluorescent signals) with the added possibility of following the same cells over time. This allows for the study of temporal differences in phenotypes. Time laps fluorescence microscopy has been utilized to demonstrate temporal heterogeneity in gene expression in *E.coli* cells, by using GFP as a reporter for swithcing on of the arabinose utilization system in the bacteria. A large variation in time from addition of arabinose to the switching on of the system was observed at low arabinose concentrations[15;16]. With the combination of a microfluidic device and time laps fluorescence microscopy, single cell observations of bacteria under varied and controlled conditions can be obtained. Such a system, where *E. coli* cells where allowed to grow in thin grooves in a microfluidic device, allowed the cells to form single line colonies (figure 3). The cells where subsequently treated with antibiotics, and after treatment normal growth medium was introduced to the surviving bacteria. In this way persister cells were located, and the arrested growth of such cells, even before antibiotic treatment, was observed[10]. The same system has been utilized to monitor the induction of fluorescent proteins[34]. Single cell time laps microscopy has also been used to study gene regulation at the single cell level, and the impact on gene expression by noise in the system could be studied[14]. The throughput of such single cell time laps microscopy techniques is naturally much lower than the sampling rates achieved in a flow cytometer, but the added dimension of time offered by these techniques allows for new insight into the dynamics of the heterogeneity of such systems.

2.3 Bacterial microarrays

A microarray can be defined as an ordered arrangement of a high number of small (micron sized) measuring sites on a flat substrate. The measuring sites typically consist of a biological probe molecule which can bind analytes in a liquid sample, and the readout is based on detecting signals from bound analytes. The appeal of the microarray is the

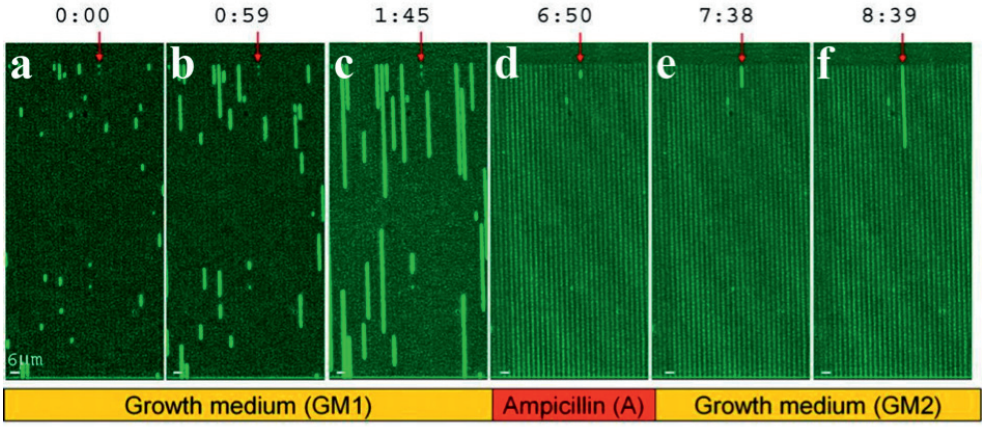

Figure 3: Images depicting a microfluidic device with grooves that allow for one-directional growth of *E. coli* cells while controlling the environment of the cells. The cells are first allowed to grow in growth medium, before an antibiotic was introduced to the system. After removal of the antibiotic growth medium was reintroduced to the system. This enabled the detection of a singel perister cell (marked with a red arrow) that displayed a slow growth rate before addition of antibiotics, but survived the antibiotic treatment and started dividing once the growth medium was reintroduced. Figure reproduced from[10] with permission.

combination of high-throughput provided by the high number of measuring sites, and the small sample volumes needed due to the miniaturization of each site.

The first widely used application of microarray technology was the DNA microarrays which rose to fame during the 1990s in response to the rapidly increasing amount of DNA sequence information that was becoming available[35]. On a typical DNA microarray, the measuring sites consist of small amounts of deposited DNA fragments, where every spot consists of different and known DNA sequences. The highly specific binding interaction between complementary strands of DNA or between DNA and RNA ensures that for each site only complementary strands to the attached DNA fragment can bind. Bound DNA or RNA fragments are identified due to their position on the array, and the binding event is usually detected by fluorescence. The intensity of the fluorescent signal can also be used as an indicator of the concentration of bound DNA or RNA fragments. DNA

arrays are widely used to measure gene expression levels, and can perform simultaneous analysis of an entire genome in a single experiment. They have been used for genotyping and to detect subtle sequence variations that are of use for disease diagnosing, evaluation and drug development[36–40] .

The success of the DNA microarrays has inspired the development of a large number of different microarray platforms, with different readout strategies, different measuring site probes and different intended use. Protein microarrays have for instance been used for screening of protein-protein or protein-ligand interactions and for evaluation and diagnosis of disease susceptibility and progression and for the discovering of potential therapeutic drugs in a more effective manner[41;42]. The micro array platform has also been developed to accommodate for tissue[43;44], cells[45] and microbes[46;47].

The controlled arrangement of bacteria offered by bacterial microarrays is of interest in many fields - from integration of live bacteria in micro- and nanoelelectromechanical systems[48;49] to sensitive detection mechanisms for bacteria[50]. The high number of measuring sites makes bacterial microarrays ideal platforms for either screening or collecting statistical information on a large number of single cells, and arrays of live bacteria have been suggested used for screening for new potential drugs, for detection of toxins or for more fundamental biological research. For instance, bacterial microarrays have been utilized to screen for persister cells, where droplets of medium containing bacteria were arrayed on a substrate. Once a persister was located, the cell could be removed by the use of a micropipette and further investigation of a single cell was made possible[51].

2.4 Fabrication of bacterial microarrays

A prerequisite of producing bacterial microarrays is the ability to control the placement of bacteria on a substrate. There are two main strategies that can be utilized; the bacteria can be directly deposited onto the selected substrate or the substrate surface can be patterned, either by chemicals or by 3D structures, in order to control where on

the substrate the bacteria are allowed to attach.

Producing bacterial microarrays by chemically patterned surfaces requires a combination of a substrate that inhibits bacterial adhesion and a pattern of a chemical that promotes bacterial adhesion. This can be obtained by several techniques, and in theory any surface patterning technique with the desired spatial resolution could be used. The small size of bacterial cells (typically 1 to 10 μm) limits the choice of patterning techniques to those capable of at least μm resolution if single cell arrays are desirable. This renders the technique of robotic printing onto microscope slides, which traditionally has been used to fabricate DNA micro arrays, unsuitable for fabrication of single bacterial arrays[36].

Techniques more commonly used in the semiconductor industry, e.g. photolithography or electron beam lithography (EBL), can be utilized to pattern surfaces for selective bacterial adhesion. Photolithography is a high trough put technique and can produce patterns with submicron resolution[52]. The technique has been utilized to fabricate patterned surfaces of biologically active molecules such as proteins, amines and alkanes[53;54].

Due to the short wavelength of the electrons, EBL has superial resolution compared to photolithography, but the scanning nature of the technique renders it time consuming. Spatial control of cell adhesion down to the single cell level has been obtained by patterning of poly ethylene glycol (PEG) hydrogels on microscope slides by EBL[55]. Here, PEG was cross-linked by the electron beam, and thereby a cell-repulsive PEG hydrogel was formed. The pattern of cross-linked PEG had small circular holes that allowed for subsequent bacterial adhesion.

The invention of the scanning tunneling microscope and the atomic force microscope has made it possible to pattern surfaces with high spatial resolution using the sensing tip of such microscopes in a technique called scanning probe lithography (SPL). SPL can be used destructively to modify a substrate surface, either by applying a large current between the tip and the sample surface to induce electrochemical oxidation of the surface

chemistry or by applying a large force to mechanically remove chemicals on the substrate surface. SPL can also be used constructively as a direct patterning technique. The most notable constructive SPL technique is dip-pen nanolithography in which an ink coated AFM tip is used to directly deposit molecules to a surface. Patterns from micro to sub 100 nanometers have been achieved, and a multiple of molecules have been pattern using this technique[56].

The abowe mentioned surface patterning techniques all produce well defined chemical patterns, some with resolution several orders of magnitude better than strictly required for bacterial microarray production. There are, however, some drawbacks of these techniques that are non-trivial. Photo and electron beam lithography require expensive equipment, cleanroom facilities and the fabrication methods can denature the biomolecules that are patterned[57]. The drawbacks of the SPL methods include low throughput, high cost and complicated experimental conditions. These drawbacks can be reduced by the use of micro contact printing, a technique in which elastomere stamps are utilized to deposit chemicals onto surfaces. This technique is described in more detail below.

Surface topography can also be used to control bacterial adhesion onto surfaces, and techniques such as photolitography and EBL or replica molding, often in combination with etching, can produce 3D patterns with the same size as individual cells. It has been observed that bacteria spontaneously produce ordered patterns on 3D arrays of pillars with the same size parameters as the bacteria (figure 4A)[58]. More controlled adhesion has been obtained in holed arrays with chemical coatings of the holes in order to promote bacterial adhesion (figure 4B)[49]. Arrays of live yeast cells have been obtained in holes on poly(dimethylsiloxane) (PDMS) surfaces, as a platform for atomic force microscopy studies of single cells (figure 4C)[59].

The alternative to patterning of the substrate when creating a bacterial microarray, is the utilization of tehniques that allows for direct positioning of bacteria. Several tech-

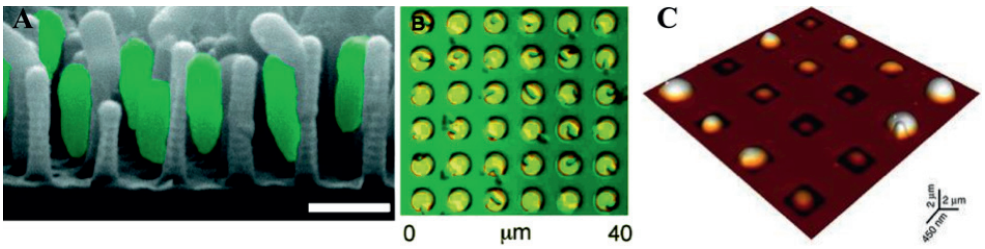

Figure 4: Illustrations of three different 3D surface patterning methods utilized for ordered capture of bacteria. A: Cross-sectional SEM image of *P. aeruginosa* cultured on structured pillars on epoxy surfaces, false-colored to highlight their orientation. The scale bar is 1 µm. B: Optical images of *E. coli* cells attached to an array of 3.0 x 0.5 µm holes prepared by electron beam lithography. C: AFM topograph of yeast cells captured in a holed PDMS microarray. Figures reproduced from[58,49] and[59] with permission.

niques commonly used for deposition of biomolecules have been adjusted to accomodate for deposition of bacteria[60–64]. Methods like contact deposition, Dip-Pen Nanolithography (DPN) and electro-dynamically generated microdroplets all deposit small droplets of bacteria in liquid solution onto predefined positions on a substrate.

The contact deposition[60] is done by dipping a pin with a narrow tip into a reservoir of bacteria in liquid, and then transferring a drop onto the substrate surface. This technique allows for parallel printing of several different types of bacteria in the same array. DPN uses the a similar approach; an AFM tip is dipped in bacterial solution and then used to pattern the surface. The electro-dynamically generated microdroplets are deposited without the use of a pin, the droplets are controlled by controlling flow rate and applied voltage. All three techniques are limited in resolution by the size of droplets they can produce, and only DPN has been used to pattern single bacteria[62].This DPN process does however require the bacteria to be suspended in an glycerol or tricine containing solution in order to deposit single bacteria since the droplet size is viscosity dependent.

Antoher technique commonly used for surface patterning of chemicals that also has been used for controlled deposition of bacteria is micro contact printing (uCP).

Elastomere stamps have been used to transfer bacteria directly onto substrates like agarose and agar for continued bacterial growth[63;64]. Weibel *et al* presented a stamp made of agarose, allowing for bacterial growth on the stamp, thereby fabricating a stamp capable of regenerating its own ink. This method is however not suitable for production of single cell microarrays. Xu *et al* demonstrated that arrays with an average of 1.4 bacteria per spot is possible using PDMS stamp based micro contact printing of live bacteria.

It is evident that the available techniques for fabrication of bacterial arrays are numerous. The optimal choice of fabrication technique will depend on the intended use of the microarray. For instance, an array designed to strictly control both the placement and orientation of motile bacteria on a surface for the integration in nanoelectromechamical systems, where the bacterial flagellum are envisioned as motors, and the bacteria thereby as their power generators has been obtained[49]. Arrays of *E.coli* with their "nose" pointing downwards and the flagellum pointing up was accomplished on 3D structured Si surfaces produced with techniques such as photo and electron beam lithography and etching. Although impressing, the specialized and time consuming techniques needed, makes such arrays unlikely instruments for use in more standard microbiology experiments.

If the goal is to develop procedures for the preparation of a bacterial microarray that can be envisioned as a simple and practical new tool in the microbiologist toolbox, this alone limits the choice of methods available. Such an array should be relatively fast and simple to produce, the substrate chosen should, for simplicity, allow for inspection by light microscopy and the chosen method of creating bacterial patterns should induce minimal stress on the bacteria. The work described in this thesis relies on micro contact printed patterns on glass substrates to promote the adhesion of bacteria to predefined spots on the substrate. The techniques used and the chemicals considered are described in more detail below.

2.4.1 Micro contact printing

Micro contact printing is a soft lithography technique, based on the use of elastomeric stamps to generate patterns on surfaces. The technique was first developed by Whitesides[65], who utilized such stamps to patterned self assembled monolayers (SAMs) on gold substrates. The technique has since been utilized to produce a variety of patterns on many different substrates[52;66–68].

The elastomeric stamps are typically fabricated from PDMS. PDMS is preferred since it is flexible, transparent and thermally cured in addition to being cheap and commercially available. The stamps are produced by mixing a prepolymer and a cross linking agent before the mixture is poured on a master mold and cured in an oven (figure 5). The master mold is made using either UV- or electron beam –lithography, depending on the wanted resolution of the pattern features. After curing, the stamp is pealed of the mold and ready to use. Both the mold and the stamps are reusable, minimizing the need for cleanroom techniques. In addition, toxic and denaturing chemicals are not needed when patterned surfaces are made with this technique. This makes soft lithography methods for many purposes more convenient, more effective and cheaper than photolithography or EBL, while still offering sub micro meter resolution[68].

There are several ways in which structured PDMS can be utilized for patterning of surfaces, but only micro contact printing will be described here. In micro contact printing (μCP) (figure 6 iv-vi) the patterned surface of the stamp is incubated with a solution containing the molecule that is to be patterned (typically referred to as the ink). After incubation the stamp is dried before it is pressed onto the substrate surface. The time the stamp is kept in contact with the surface depends on the inking chemical. Once the stamp is removed, the substrate surface is patterned with molecules that have been transferred from the PDMS structures onto the substrate.

In the work presented in this thesis, μCP has been utilized to pattern surfaces for fabrication of bacterial microarrays, as illustrated in figure 6. The surface substrate was

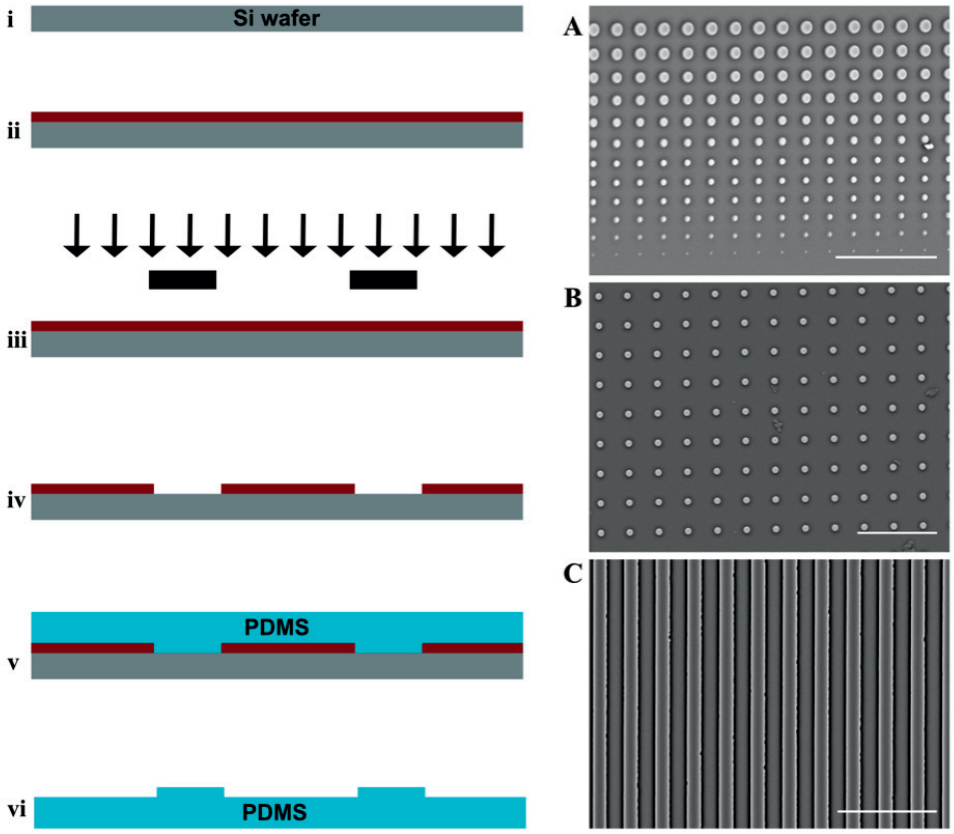

Figure 5: (i-vi):Illustration of the fabrication of a PDMS stamp; A silicon wafer is used as the substrate (i) onto which a photoresist is spun into a thin film of the desired thickness(ii). The photoresist is exposed to UV light through a mask(iii), before the photoresist is developed revealing a 3D patterned surface (iv). PDMS prepolymer and cross-linking agent is then mixed and poured onto the patterned silicon wafer(v). After thermal curing of the PDMS, the hardened polymer can be pealed off the mold and is ready for use.(A-C): Three examples of PDMS stamps used in this thesis. The stamps are coated with 5 nm gold film before imaging in a Hitachi TM3000 tabletop SEM. All scale bars are 50 µm.

first chemically modified to resist bacterial adhesion before a chemical known to promote bacterial adhesion was patterned using PDMS stamps. Bacteria in solution incubated on such patterns should only be able to adhere to the areas covered with the chemical

known to promote bacterial adhesion (figure 6 viii-ix). The choice of chemicals used to modify the substrate are discussed below.

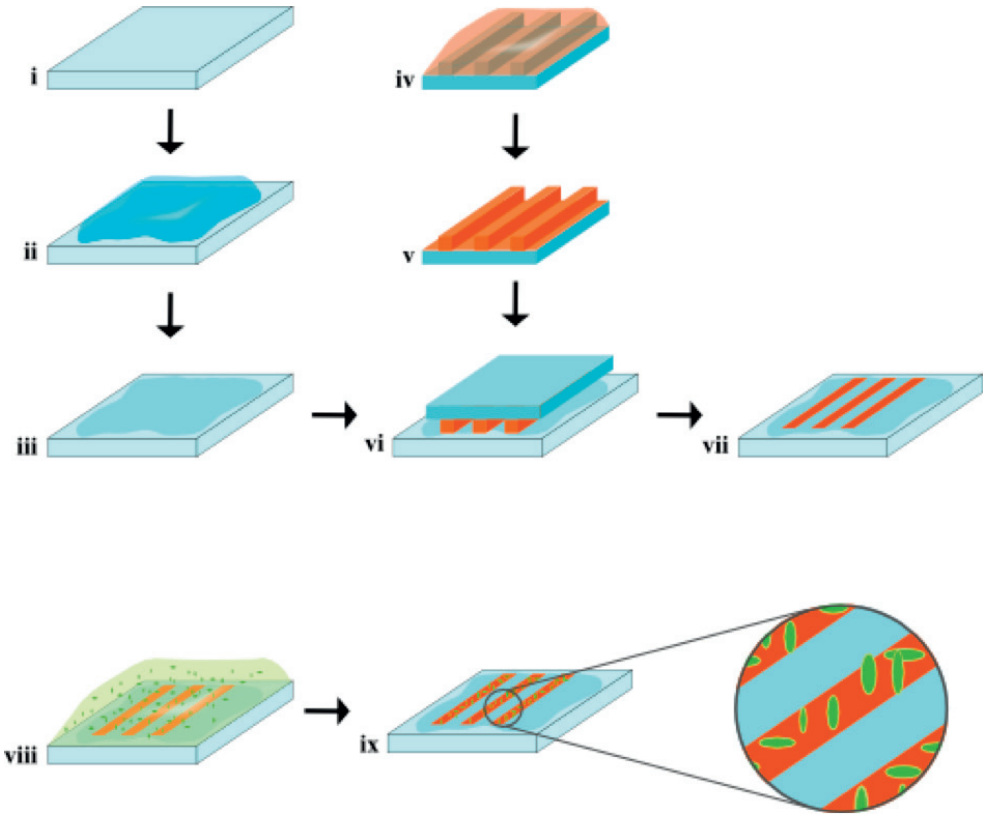

Figure 6: Illustration of preparation of patterned surfaces for selective bacterial adhesion onto predefined areas on the substrate. i-iii: A cleaned glass substrate is coated with a polymer known to resist bacterial adhesion. iv-vi: A PDMS stamp is immersed in a solution containing a chemical known to promote bacterial adhesion, before excess ink is dried off and the stamp is placed pattern side down on the polymer coated glass substrate, giving rise to a chemically patterned surface (vii). viii-ix: Incubation of live bacteria in liquid solution on the patterned substrates, with subsequently rinsing off of non-adhered bacteria, results in bacterial adhesion only to selected areas of the glass substrate.

2.4.2 Surface modification for selective bacterial adhesion

Fabrication of bacterial miroarrays based on chemically patterned substrates require a substrate that inhibits bacterial adhesion everywhere except for the areas the bacteria are intended to adhere to. Bacteria are known for their ability to adhere to almost any surface, and given enough time, subsequent biofilm formation will occur[69–71]. Surface treatment of the array substrate is therefore needed in order to control the bacterial attachment. Prevention of biofilm formation is of interest in areas as divers as medical technology, food production or marine technology and a wide variety of ways of avoiding the initial adhesion of bacteria have therefore been suggested[72;73]. However, chemical coatings of polyethylene glycol (PEG) or polyvinylalcohol (PVA) stands out as readily available, easy to use and non-toxic surface treatments.

PEG-based coatings are commonly used as antifouling coatings[72], and their resistance to bacterial adhesion is thought to be due to their exceptional protein resistance. In this thesis glass surfaces were PEGylated with using a grafted polymer with a backbone of poly-L-lysine (PLL) onto which PEG is grafted in a comb-like structure (PLL-g-PEG). This PEGcopolymer was designed for easy PEGylation of negatively charged surfaces[74], and offers simple immobilization of PEG on glass through electrostatic interactions between the positively charged PLL polymer and negatively charged glass substrate. Surfaces PEGylated with PLL-g-PEG have low protein adsorption[74;75], and have successfully been used as a passivation layer for patterned adhesion of several types of eucaryotic cells[76–78]. Once immobilized on glass surfaces, the PLL-g-PEG copolymer has been shown to be stable on glass for months when stored in air at room temperature[78].

PVA hydrogels are known to resist adhesion of proteins, cells and bacteria[79–81]. For long time adhesion of the PVA hydrogel to a substrate, adhesives like glutaraldehyd or polydopamine are required in addition to thermal annealing[82]. For short term aspects, thermal annealing of the hydrogel is sufficient, although this renders the hydrogel fragile

and easily damaged[82]. For strict control of PVA film thickness, the PVA hydrogel can be spun coated onto the substrate surface[81].

The protein Bovine serum albumin (BSA) has also been used to reduce non-specific protein adsorption to surfaces, and is therefore a candidate for surface treatment of glass to avoid bacterial adhesion. BSA has a net negative charge at physiological pH which could repel negatively charged bacteria through electrostactic interactions[83]. It has also been shown that μCP lattice patterns of BSA inhibits bacterial adhesion given that the lattice parameter are smaller than the size of the bacteria[84]. The anti-adhesion effect of BSA has been explained by steric repulsion forces exerted by BSA. BSA has however been known to interact with bacteria, and some adhesion has been observed[85].

After surface treatment of the array substrate to avoid bacterial adhesion, a pattern of bacterial adhering molecules can be transferred onto the substrate. Although bacteria are known to be able to attach to a variety of surfaces, through a variety of strategies[70;86;87], some over all strategies can be suggested to promote bacterial adhesion. The vast majority of bacteria have a negative surface charge in aqueous solutions. Patterns of positively charged polymers are therefore not a surprising choice for patterned adhesion. Polymers such as poly-L-lysine (PLL) and polyethyleneimine (PEI) have successfully been utilized to attach both bacteria and eukaryotic cells to substrates[48;88–90]. A different option is the use of polydopamine (PD) patterns. Dopamine is a chemical analogue to 3,4-dihydroxy-L-phenylalanine (DOPA) which is among the chemical motifs significant to the adhesion properties of a group of proteins produced by mussels[91]. This family of proteins, often referred to as mussel adhesive proteins, or MAPs, are the main reason for the mussels ability to attach to surfaces both in salt and fresh water conditions and often under stress from strong water currents. Dopamine is inexpensive and commercially available, often as dopamine hydrochloride, and is easily polymerized into PD. PD is chemically versatile and it has been suggested that it can interact through π-stacking, charge transfer and hydrogen bonds[92;93]. This chemical versatility enables

PD to stick to a variety of surfaces. PD is also rich in catechol and amine groups which can bind amine, thiol and catechol moieties in addition to metal ions and particles. This has, in addition to PDs very low toxicity and high degree of biocompatibility, led to an interest in using PD to pattern both eucaryotic and bacterial cells[81;94].

2.5 Microfluidics, cell encapsulation and the microarray platform

Recently, there has been an increased interest in growing cells and microorganisms in 3D polymer hydrogels instead of on the surface of traditional two-dimensional culture dishes[95]. The 3D structure of the gels can more closely mimic the natural environment of the cells, which is especially important for eukaryotic cells that naturally exists in tissue with 3D structure. The porous nature of such gels allows for the diffusion of nutrients and oxygen into the gels while waste diffuses out. By reducing the size of the gels to the micrometer scale, the sample volumes needed are reduced, while encapsulation of single microorganisms remains possible. Encapsulation of cells in polymer microgels is therefore of interest for numerous studies within the field of biology or tissue engineering.

Several techniques are available for the fabrication of microgels, and several types of hydrogels are commonly utilized for the encapsulation of cells. These techniques includes molding[96], multifluidic electro static spraying[97] or laser direct writing[98;99]. The polymer utilized can be either natural polymers such as gelatin[100] or collagen[97] or synthetic polymers such as PEG or poly(lactic acid)[101]. The system utilized in this thesis is however based on gelling of alginate in a microfluidic device.

Alginate is a naturally occurring polysaccharide consisting of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues. The relative amount of the two uronic acid monomers as well as their sequential arrangement along the polymer chain differs widely, depending on the origin of the alginate. Alginate can form a hydrogel through ionic cross linking with divalent cations, and the properties of the alginate hydrogel can be tuned by varying the M and G content of the polysaccha-

ride polymer. Alginate hydrogels are biocompatible and has several uses in biomedical applications[102].

The field of microfluidics is based on controlling and manipulating the flow of small volumes of liquid constrained in geometrical structures with features on the milli- to micro meter scale. The small length scales give a dual benefit in that required sample sizes are minimized and that the liquid flow in the devices is laminar rather than turbulent. Under laminar flow fluids flowing parallel to each other will not mix as they would under turbulent flow since molecular transport between the two fluids are driven by the relatively slow process of diffusion. The laminar flow regime in these devices can be utilized for e.g. control over concentration gradients[103] or for droplet formation[104].

Droplet formation in microfluidic devices can be performed using pressure driven strategies, in which a water phase brakes up into droplets in a oil phase. Three commonly used device geometries utilized to accomplish this are co-flow, T-junctions or flow focusing, as illustrated in figure 7. The details of these droplet formation techniques are reviewed elsewhere[104]. Common for these three geometries is droplet formation as a result of competing stress; surface tension between the two phases tries to reduce the inter facial area while viscous stress tries to drag and extend the interfacing segment downstream. This results in a homogeneous size distribution for the produced droplets. Such droplet formation in microfluidic devices can be utilized for cell encapsulation by adding live cells to the water phase, resulting in cell loaded water drops in the oil phase. These cell loaded droplets can be utilized for single cell experiments[105;106].

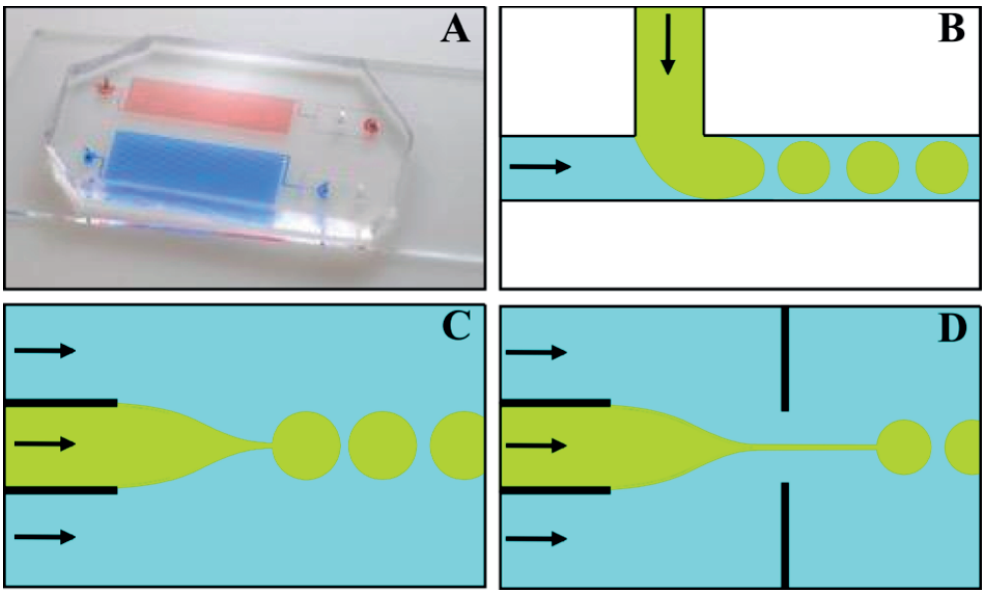

Figure 7: A: an example of a simple microfluidic device (image used with permission of the microfluidics group at NTNU). B - D: Three different geometries utilized for droplet formation in microfluidic devices: T-junction (B), co-flow(C) and flow focusing (D).

By replacing the water phase in such droplet fabricating devices with an aqueous solution containing a water soluble polymer such as alginate, the droplets formed can be gelled after droplet formation, and the microfluidic devices can thus produce cell-loaded microgels[107]. Unlike cell-containing water droplets in oil, the cell loaded microgels can be removed from the oil phase after fabrication and be stored in physiological buffers. Such cell loaded microgels are an interesting platforms for single cell measurements, but also as building blocks for tissue engineering[101].

Encapsulation of cells by utilization of microfluidic devices has several advantages compared to other methods; the reagent volumes needed are small, the throughput is high, the droplet sizes that can be produced are in the micrometer range and the droplets have a size distribution with a narrow polydispersity. In addition, with clever design of the microfluidic device, there are several morphologies available, including e.g. uniform

droplets, a core/shell structure[108] or janus beads[109]. The system also offers physical and chemical isolation and thereby eliminates the risk of contamination. Lastly, microgel fabrication can be achieved on inexpensive, dust free and disposable devices[104;105;110].

There has been an interest in combining the advantages offered by small 3D microgel cultures with the high throughput of an array platform. The microgels ensures a suitable microenvironment for the encapsulated cells, while the array display enables easy detection and simultaneous inspection measurements of a large number of microgels. Arrays of cell loaded microgels can be envisioned for large scale single cell measurements, as screening platforms or for toxcicity or drug testing[111]. However, organizing the microgels into structured arrays is not trivial. The microgels can be captured in 3D structures, e.g. in holes on a flat substrate[97] or in u-shaped traps in microfluidic devices[112]. By choosing alternative fabrication methods to microfluidics, the microgel polymer can be patterned first and subsequently gelled "on site"[113] or laser direct writing of polymer microgels can be applied to gel and direct placement in the same step[98;99]. Such techniques are however somewhat complicated, and not all offer single cell resolution. An alternative method for simple organization of microgels is therefore needed. In this theses a method of immobilizing alginate microgels onto glass substrates is suggested. Inspired by the fabrication method of bacterial microarrays, glass substrates are PEGylated before a chemical pattern is introduced using micro contact printing. By printing of the positively charged polymer PEI, the negatively charged alginate microbeads where easily immobilized through electrostatic interactions.

3 Summary and discussion of papers

3.1 Brief summary of papers

Paper I

Micro contact printing was utilized to pattern surfaces for the fabrication of arrays of live *Pseudomonas Putida* (*P. Putida*) KT2440. Different combinations of polymers known to prevent bacterial adhesion and chemicals that promote such adhesion were evaluated to find a combination that gave a high degree of bacterial adhesion to patterned areas, while leaving the rest of the substrate free of bacteria. A combination of a PEGylated glass substrate with patterns of deposited PD was found to be effective for preparation of *P. Putida* KT2440 microarrays. Once the optimal chemicals for surface functionalization were identified the parameters of the microcontact printed patterns where evaluated. PD patterns of circular spots of different diameter and different separation distances where printed onto PEGylated surfaces. Bacteria were incubated on the patterns, and the number of spots with attached bacteria, along with the number of bacteria on each spot were determined for each of the different spot size. Based on the results, a new array pattern with spots of 3.5 μm in diameter separated by 15 μm, was designed and utilized to produce arrays of *P. Putida* cells. These arrays were shown to have a high degree of coverage (bacteria were attaced to between 97 and 100% of the spots) and a low number of bacteria on each spot. A live/dead assay showed that 99.1% of the attached bacteria were alive, and a time series performed on arrays of *P. Putida* cells showed that the immobilized bacteria both divided and produced GFP upon induction.

Paper II

In this paper, highly ordered bacterial microarrays were prepared based on the method developed in Paper I, and used to study the heterogeneity within a bacterial population. The results obtained related to population heterogeneity were compared to the results

obtained using the more established flow cytometry approach. To different strains of *P. putida*, differing in the plasmid they carried and thereby the inducer needed to induce GFP production were studied. The fluorescence intensity was determined as a function of time from added inducer for several different inducer concentrations using both a flow cytometer and image analysis of time-laps micrographs of bacterial microarrays. The average fluorescence as a function of time showed the same observed tendency using both measurement techniques. The same holds for the distributions of GFP intensity for different times and inducer concentration. By analyzing time series of single bacteria on the arrays, a large inter-cell difference in fluorescent intensity was detected. The variation in fluorescent intensity between cells was significantly larger than any variation over time in any one bacteria, and individual cells were found to follow the same percentile within a population over time.

Paper III

In this paper we encapsulated three different microorganisms in alginate microgels using a microfluidic device. The microgels were subsequently immobilized in a arrray pattern on microcontact printed PEI patterns on PEGylated glass substrates. We demonstrated that patterns of the positively charged polymer PEI on PEGylated glass surfaces, offers a simple approach for selective immobilization of negatively charged alginate microgels. The microgel arrays were shown to be a practical platform for following the growth of microorganisms over time. Two of the microorganisms used in this study, (*Synechocystis sp.* PCC 6803 and *Chlamydomonas reinhardtii* CC-4532) have doubling times of approximately 12 hours. To follow the growth of these organisms inside arrayed microgels, the arrays were imaged every 24 hours and stored in tempered incubators in between imaging. The array format of the immobilized microgels facilitated easy identification of the same microgels the next day. In addition, the microgel arrays were suggested as a platform for screening and subsequent removal of microgels containing cells of interest.

This was demonstrated by making arrays of microgels loaded with two different strains of *P. Putida* KT2440, differing only in the inducer needed to induce GFP production. Cells induced by arabinose could be detected and differentiated from cells not induced by arabinose, by the appearance of fluorescence after addition of the inducer to the medium. A microgel containing a fluorescing bacterium was removed from the array using a micro pipette. The microgel was subsequently suspended in medium and incubated over night. After 24 hours a high number of bacteria existed in the medium, and addition of arabinose induced GFP production in the bacteria, thereby confirming that the colony in the test tube consisted of *P. Putida* carrying the plasmid that produces GFP upon the induction of arabinose.

3.2 Discussion

The aim of this thesis has been to develop a bacterial microarray platform using relatively simple fabrication methods that are applicable in standard microbiology laboratories. With that in mind, μCP was chosen as the preferred method of introducing chemical patterns to glass substrates in order to produce microarrays onto which selective adhesion of bacteria can occur. In paper I and II the successful immobilization of *P. putida* KT2440 on μCP patterns of PD on PEGylated glass was both presented and utilized for inspection of heterogeneity in gene expression. In paper III the same surface functionalization techniques were utilized to produce arrays of cell-loaded alginate microgels.

In paper I several aspects of fabricating bacterial micrarrays were evaluated, most importantly both a chemical regime that ensured selective bacterial adhesion and the pattern parameters needed for single cell attachment were assessed. The pattern parameters chosen were a compromise between wanting a high fraction of spots with attached bacteria while at the same time minimizing the number of bacteria attached to each spot. In paper II, eight patterned substrates where utilized for inspection of attached

bateria. For these microarrays, the fraction of covered spots varied between 98% and 85%, and the average number of attached bacteria on each spot was found to be 2.3. This is a degree of coverage that is acceptable for the proposed use of the microarrays as the number of spots with attached bacteria is high enough for collection of statistical information on the bacteria. The average number on each spot is, however, slightly higher than the ideal of 1 bacteria per spot. A slight reduction in the spot diameter, for instance down to 3 μm, which reduces the spot area with approximately 15 %, could reduce the average number of bacteria per spot without decreasing the coverage to an unacceptable level. It is however important to remember that we are attempting to immobilize bacteria by incubation on surfaces that promotes adhesion to specific areas. This "self-adhesion" of motile and living bacteria this will always give the system a degree of variability that it is impossible to control for. An array with an average close to one bacterium per spot should be realizable by adjustment of the pattern parameters, but the perfect array with thousands of spots, each with one single attached bacterium very well might be unattainable with the fabrication methods utilized in this thesis.

In paper I, PD patterns on PEGylated surfaces was shown to be very effective for immobilizing *P. putida* KT2440. In paper III, PEI was utilized for selective adhesion of several microorganisms. Determining a chemical regimes that allow for the selective adhesion of a wide range of bacteria, especially for model organisms such as *E. coli*, is of interest for the further development of this platform. The PEGylation of glass surfaces utilized here is likely to resist adhesion from most microorganisms, given its common use in the literature[72]. The existence of a universal "glue" for promotion of adhesion of bacteria is however unlikely considering the vast variety of bacterial species that exists. A range of different adhesion promotion molecules applicable for different species should therefore be identified.

As demonstrated in article II, time laps imaging of bacterial microarrays provides statistical information about the attached bacteria that is comparable to that of a flow

cytometer. The data collection from the microarrays was preformed by a confocal microscope. The fluorescent signals collected were from bacteria that are approximately 1 μm thick, which gives a relatively thin section of the array from which fluorescent signals can be collected. To ensure that differences in fluorescence intensity detected can be related to differerences in GFP-production in the cells the sample holder on the microscope must ensure the flatness of the array during imaging. A tilt of 1 μm from one side of the imaged section to the other will give differences in fluorescence not related to rates of GFP production but to the fact that some bacteria are slightly out of focus. To correct for this we chose to collect a z-stack of images and merge the images based on maximum fluorescence in each image. A automated system with a sample holder with piezoelectric control on both z-height and tilt in combination with software for adjustment of the sample tilt wold probably correct for this fact to a grater degree than we were able to.

The program developed for extraction of information from the time laps images was utilized to facilitate simple counting of bacteria related to their position on the arrays and thereby gave statistics on the efficacy of the developed fabrication method. The main purpose of the program was, however, to extract information on the fluorescence intensity of each cell for comparison with data collected by flow cytometry. For comparison purposes, the fluorescence for each cell was defined as the sum of pixel intensities of the pixels that make up each bacterium. This does however not take into account the size of the bacterium and as such a small and bright bacterium can get the same intensity value as a larger but more dim bacterium. This was done to more closely resemble the data collection in a flow cytometer where the intensity measured is not directly coupled to bacterial size. The size of the bacteria can easily be accounted for with data acquired on the array platform by simply calculating the average of the pixels in each bacterium in stead of the sum. The time laps series contains information on a range of parameters coupled to the cells, and which information one wants to extract will be

related to the experiment performed. For instance, the rate of division could be coupled to fluorescence intensity to investigate whether or not the more bright bacteria also are the fastest growing cells.

Paper III describes immobilization of alginate microgels containing microorganisms onto functionalized surfaces. This immobilization is based on interactions between the stamped PEI patterns and the alginate microgels, rendering this immobilization method independent on the microorganisms within the microgels and thereby avoiding having to identify a suitable adhesion promoting chemical for the microorganism in question. For this system, a slight tilt of the array will not cause the same problem as for the bacterial arrays, since the microgels are aproximately 50 μm in diameter. One must however be carefull when searching for fluorescent signals from microorganisms within the beads since the cells will be positioned at different height within the beads and a fluorescent cell will appear non-fluorescent when not in the focus plane of a confocal microscope.

Lastly, the arrayed microgels were suggested as a method for both localization and isolation of cells within alginate microgels. The microgels could be removed from the array using a micropipette connected to a micromanipulator and the microgel could subsequently be cultured. The possibility of removing single cells from the bacterial microarrays described in paper I and II, where the cells are directly immobilised onto the surface, for further studies would be a very interesting further development for the platform. The arrayed microgels are relatively simple to remove by the aid of a micromanipulator, and the microgel protects the cells from mechanical stress during the removal. The much smaller size of single bacteria might present a challenge along with the fact that the bacteria themselves are attached to the substrate and not protected by an alginate microgel. Hoewer, a micromanipulator with sufficiently good control of movements in the x, y and z direction can be utilized to collect single cells, as has been demonstrated by others[51;114;115].

4 Conclusion and outlook

This thesis demonstrates that micro contact printing of chemicals onto PEGylated glas surfaces is an effective and simple method for fabrication of both bacterial microarrays and arrays of cell loaded alginate microgels. The fabricated bacterial microarrays of *P. putida* KT2440 where used for time laps imaging, and subsequent data analysis was utilized to gather statistical information on heterogeneity in gene expression in the bacteria. The results obtained were comparable to data attained using flow cytometry, showing that the arrays gives qualitatively comparable results to a flow cytometer. The advantage of using time laps imaging of microarrays over flow cytometry lies in the possibility of following the same bacteria over time. This enabled the extraction of additional data on the behavior of the bacteria, we could for instance show that single bacteria tended to stay in the same percentage of the fluorescence distribution over time. Such information is unattainable in a flow cytometer.

The microarray platform was also adapted to allow for the immobilization of cell loaded alginate microgels. The arrayed microgels constitute a platform for immobilization of cells independent of the cell type, as the attachment to the array pattern is solely dependent on the interactions between the PEI pattern on the array and the alginate microgel. As a platform, the arrayed microgels allows for the study of 3D cultures over time as well as screening and subsequent removal of single microgels by utilization of a micropipette.

The techniques utilized in this thesis are purposely chosen for their relative simplicity in use and relative low cost. In order for this array platform to be utilized by microbiologist, it is important that the need for specialized equipment and labs is minimized and to ensure that this can be implemented in a standard lab. This is also a versatile system, as the patterns and chemicals used can be optimized for each experiment.

For further development of the system, identifying combinations of chemicals onto which commonly used model organisms such as *E.coli* can be selectively patterned is of importance. Another interesting development would apply both array platforms presented in this thesis for studies of culture growth. In the 3D microgels, the colony can expand in all three dimensions while the colony is contained within the bead. This greatly reduces the probability of cells to release into the medium, but only until the colony outgrows the bead. The 3D nature of the colony does however requires timely z-stacks of images in order to image all the cells within the colony. Alternatively, colony growth can be studied on the two dimensional microarrays. Here, the cells grow outward on the surface and a single image will capture all bacteria in the colony. This approach does however increase the probability of the escape of new cells to the medium. This could possibly be avoided by the addition of a thin alginate film on top of the arrayed bacteria

Lastly, microfluidics is becoming a much used technique for studies of microorganisms, and there is often a need for immobilization of microorganisms also in such devices. The result achieved here is thereby also relevant for implementation in microfluidic systems. Combining the microarray with a microfluidic device to control the liquid flow over the arrays could give very good control over the bacterias environment, and it will be possible to flush out any detached and freely swimming bacteria.

Thus, the microarray platform presented in this thesis is a simple yet versatile tool for inspection of both bacteria and cell loaded microgels that can be developed into a very useful tool for microbiologists.

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Paper I

PLOS ONE

RESEARCH ARTICLE

The Design of Simple Bacterial Microarrays: Development towards Immobilizing Single Living Bacteria on Predefined Micro-Sized Spots on Patterned Surfaces

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Abstract

In this paper we demonstrate a procedure for preparing bacterial arrays that is fast, easy, and applicable in a standard molecular biology laboratory. Microcontact printing is used to deposit chemicals promoting bacterial adherence in predefined positions on glass surfaces coated with polymers known for their resistance to bacterial adhesion. Highly ordered arrays of immobilized bacteria were obtained using microcontact printed islands of polydopamine (PD) on glass surfaces coated with the antiadhesive polymer polyethylene glycol (PEG). On such PEG-coated glass surfaces, bacteria were attached to 97 to 100% of the PD islands, 21 to 62% of which were occupied by a single bacterium. A viability test revealed that 99% of the bacteria were alive following immobilization onto patterned surfaces. Time series imaging of bacteria on such arrays revealed that the attached bacteria both divided and expressed green fluorescent protein, both of which indicates that this method of patterning of bacteria is a suitable method for single-cell analysis.

Introduction

The awareness of the challenges connected to population averages, i.e. their inherent masking of the behavior of minority subpopulations, explains why single-cell analysis is increasingly used in multiparametric analysis of microbial cells [1, 2]. Single molecule studies have revealed that a major strength of studying processes at the level of individual cells lies in the direct measurement of distributions of properties, rather than their ensemble averages [3, 4]. This awareness is in the biological research community accompanied by a growing demand for sensitivity and throughput in single-cell studies. For many purposes, the possibility to correlate the behavior of an individual cell prior to, during and after changing its environmental conditions is also required. High resolution temporal imaging of bacterial microarrays allows a high number of

individual bacterial cells to be followed over time [5]. This approach thus allows for insight into overall population behavior as a function of time.

A bacterial microarray can be defined as a supporting material onto which bacteria are attached in a regular and well defined pattern. Different strategies have been proposed for the preparation of bacterial microarrays. They can be divided into two main categories. The first category includes strategies where the bacteria are deposited directly onto the substrate in a predefined pattern. The second category is characterized by the use of surface patterning techniques allowing the surface to be patterned in such a way that bacteria only attach to specific areas of the pattern.

Many of the studies belonging to the first category rely on deposition of droplets containing the bacteria [6–10]. They are therefore limited in resolution by the size of the droplets that can be deposited, and only dip-pen nanolithography (DPN) has been used to deposit single bacteria [8]. DPN based deposition of single bacteria does however require the bacteria to be suspended in a glycerol or tricine containing solution since the deposited droplet size is viscosity dependent. Another limitation of this approach is connected to the requirements for dedicated instrumentation to make each array, complicating the possibility for mass production. Alternatively, bacteria can be directly deposited using microcontact printing (μCP) [9, 10]. μCP is a simple, fast and reproducible way of patterning large areas (up to cm^2) on a substrate with few restrictions on the substrates available for patterning [11–13]. However, using μCP to deposit bacteria entails a risk of harming the bacteria due to exposure to altered environmental conditions during the stamping process.

The second category of approaches, i.e. allowing bacteria to attach to predefined spots on a patterned surface, minimizes the direct handling of bacteria and the risk of exposing them to air. Surface patterning involves either chemical or topographic micro scale patterns on a chosen substrate. Surfaces with pillars in the same size range as a single bacterium have been shown to produce regular patterns of bacteria [14]. Single *E.coli* cells have been successfully immobilized in holed arrays on a silicon substrate [15]. The production of topographical patterns does however require the use of time consuming lithographic techniques and access to cleanroom facilities. Chemical patterning is commonly obtained by μCP which has successfully been used for patterning of surfaces for selective adhesion of bacteria. Single bacterial arrays have been achieved by using both gold coated silicon oxide wafers [16] and glass substrates [17, 18]. When aiming at optimizing the bacterial microarray technology for use in biologically oriented laboratories, the possibility of preparation on transparent microscopy slides is an advantage, and this requirement conflicts with the use of gold coated silicon oxide wafers. Furthermore, the need for modification of the bacteria to be immobilized, in order to introduce reactive surface groups to be used for the immobilization [17] restricts the applicability of the technique. This restriction has been overcome by altering the chemicals used for the bacterial adhering areas of the patterned glass surfaces [18]. The chemicals used in producing these arrays are, however, classified as hazardous. In addition, the glass surfaces must be activated by oxygen plasma before patterning, which requires equipment that is not standard in an ordinary biology lab. Further optimization of the experimental approach for production of bacterial microarrays is therefore needed.

A general way of immobilizing bacteria is to pattern positively charged polymers on a substrate. Most bacteria are negatively charged, and will bind to such polymers through electrostatic interactions. Commonly used positively charged polymers are polyethyleneimine (PEI) and poly-L-lysine (PLL), which have both been used to immobilize bacteria [16, 19]. PEI has been reported to give higher viability to the attached bacteria when compared to PLL [19]. A higher concentration of PLL improved adhesion at the cost of more induced stress in the attached bacteria [20]. Another approach for immobilizing bacteria relies on the use of poly

(dopamine) (PD). Dopamine and its analogues are an essential part of the adhesive proteins that mussels use to attach to a variety of surfaces under wet conditions [21]. PD has been shown to produce a thin film which has also proven itself to be very useful for binding of molecules [22], giving rise to the interest in using PD for immobilization of both eukaryotic and bacterial cells [23, 24]. Bacteria can also be immobilized by patterning antibodies for the specific bacteria [25], or through streptavidin—biotin interactions provided that the cell-surface proteins of bacteria are chemically biotinylated [17].

To avoid unspecific adhesion of bacteria to areas that are not functionalized with bacterial adhering chemicals, the substrate is often coated with a passivating chemical. Polyethylene glycol (PEG), bovine serum albumin (BSA) and poly(vinyl) alcohol (PVA) are known to prevent protein adsorption when coated on surfaces, and are therefore used to inhibit bacterial adhesion. A lattice of BSA printed on glass cover slips has been shown to inhibit *E. coli* adhesion when the lattice features where smaller than the bacteria [26]. PEG is commonly used in order to avoid bioadhesion [27–29], and has also been useed in combination with PD to pattern *E. coli* on polystyrene surfaces [23]. PVA hydrogels have been shown to resist protein adsorption [30] and have been used in studies aimed at making patterns of eukaryotic cells [24, 31].

In this paper we propose an approach for the preparation of bacterial microarrays using μCP of bioadhesive chemicals to glass substrates coated with antiadhesive chemicals in order to selectively immobilize bacteria onto predefined spots on the substrate (Fig 1a). In this study *Psaudomonas putida* KT2440 was used, which is a non-pathogenic bacterial strain that has a GRAS (generally regarded as safe) status. They are suitable bacterial bio-platforms due to their metabolic and stress-endurance properties [32]. The design features of the elastomer stamps are evaluated to optimize the probability of capturing single bacteria on the adhesive spots of the array.

Materials and Methods

Stamp production

The master mold for stamp production was produced by photolithography. A 4″ silicon wafer (Siltronix) was spincoated with the positive photoresist Microposit S1818 (Microresist Technology) before exposure to UV light through a quartz mask (Computographics) for the desired pattern. The photoresist thickness was 2.3 μm, resulting in stamp features of that hight. Three different patterns where used (Fig 1). The first pattern consists of slits of width 5 μm interspaced by 5 μm opaque lines (Fig 1d). The second pattern consists of 13 circular holes of diameter increasing from 0.8 μm to 4.4 μm on an opaque background (Fig 1a, left side). This pattern was produced in four versions, each characterized by a vertical separation distance d_1 of 3, 4, 6 or 8 μm between the circular holes and a fixed horizontal distance, d_2, between the center of each hole of 7.4, 8.4, 10.4 or 12.4 μm. The third pattern consists of circular holes with a diameter, d_h, of 3.5 μm with a separation distance between the circular holes equal to either 10 or 15 μm (Fig 1a, right side). After development, the wafer was covered by PDMS (Sylgard 184 from Dow Corning). A 1:10 weight solution of PDMS curing agent to base was used for pattern one and two. A 1:5 weight solution of curing agent to base was used for the third pattern for a stiffer PDMS to avoid roof collapse of the stamps due to the larger separation distance between the pillars of these stamps. The PDMS was cured on the master in an oven for 2 hours at 80°C. After curing, the stamp was peeled off the master and was ready for use. Some of the stamps were imaged using a TM3000 Hitachi tabletop SEM. Prior to SEM inspection the stamps were sputtercoated with a 20 nm thick gold coating using a Cressington 208 HR B sputter coater.

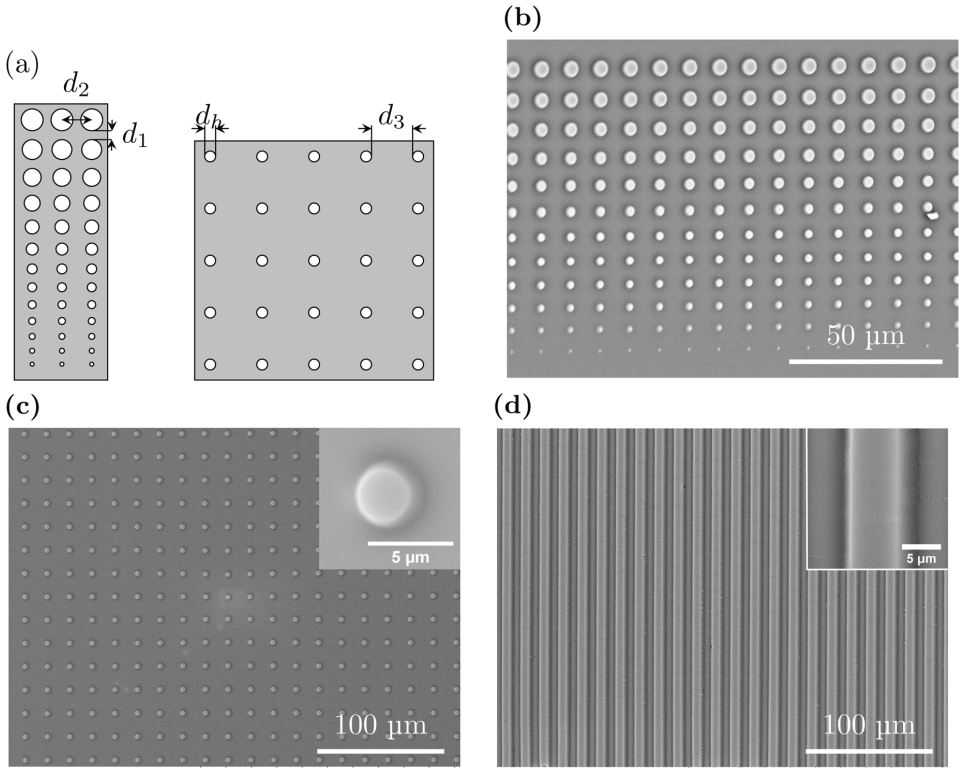

Fig 1. (a): The patterns on the photolithography masks used to produce PDMS stamps. The first pattern (left) consisted of 13 circular holes of diameter increasing from 0.8 µm to 4.4 µm on an opaque background. The mask contained four quadrants, each characterized by a vertical separation distance d_1 of 3, 4, 6 or 8 µm between the circular holes and a fixed horizontal distance d_2 between the center of each hole of 7.4, 8.4, 10.4 or 12.4 µm. The pattern on the second photolithography mask (right) consisted of circular holes with a diameter d_h of 3.5 µm with a separation distance d_3 between the circular holes equal to either 10 or 15 µm. (b), (c) and (d): SEM micrographs of gold coated PDMS stamps intended for patterning of glass surfaces by µCP. The stamps shown in (b) and (c) are produced using the photolithography masks schematically illustrated in 1(a). The stamp depicted in (d) was obtained using a photolithography mask with slits of width 5 µm interspaced by 5 µm.

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Patterning of glass surfaces using µCP and PDMS stamps

The surface patterning technique µCP was used to introduce circular spots or lines coated with chosen chemicals introducing the surface properties needed in order to obtain bacterial arrays. The PDMS stamp was incubated with a drop of the selected chemical (10 to 30 minutes) followed by blow drying with nitrogen and placed pattern side down on the substrate to be patterned. A pressure was applied onto the PDMS stamp throughout the stamping period by placing a weight of 100 grams ontop of the stamps, in order to obtain good contact between the features of the stamp and the substrate.

The reproducibility of the µCP process was investigated by stamping cleaned glass cover slips (borosilicate glass, VWR international) employing PDMS stamps incubated in a solution containing qdot 655 ITK amino (PEG) quantum dots (Life Technologies) diluted in MilliQ

water to a concentration of 10 nM. The patterns where imaged with a Zeiss 510 Meta microscope with a 20x objective (NA = 0.5, liquid). The size of the introduced quantum dot coated areas was determined using the analyze particles function in ImageJ software, and the diameter was calculated based on these results.

For patterning of surfaces intendedused for preparation of bacterial microarrays, a Willco-dish kit (Willco Wells) was used. The dish facilitates covering the microarrays in liquid during investigation, and this kit allows for patterning of the glass bottom of the Willco-dish before assemblement of the dish. Prior to being patterned, the glass surfaces were cleaned by immersion in a 1:1 V/V solution of puriss grade hydrochloric acid (Sigma-Aldrich) and methanol (Sigma-Aldrich) for 20 minutes before rinsing in MilliQ water and drying by a stream of nitrogen gas. To avoid bacterial adhesion the glass surfaces were passivated through coating with the chemicals BSA, PVA or PEG prior to patterning using μCP. The coatings were introduced using the following procedures: BSA (Sigma) was dissolved in phosphate buffered saline (PBS, Sigma) to a concentration of 1 mg/mL and added to the glass surface for incubation for 20 minutes. After incubation the glass surface was rinsed in MilliQ water and dried by a stream of nitrogen. Coating with PVA was obtained by dissolving 22 kDa poly(vinyl) alcohol (PVA) from BDH Chemicals to 1 wt % in MilliQ water and spincoating this onto on the glass surface before curing on a hotplate at 130°C for 30 minutes. PEGylation of the surfaces was acheived by immersion for 60 minutes in a solution containing poly-L-lysine (20 kDa) grafted with PEG(2 kDa) (in the further referred to as PLL-g- PEG) from Susos was dissolved in MilliQ water to a concentration of 1mg/mL. After incubation the excess liquid was removed and the glass was rinsed in PBS before rinsing in MilliQ water and dried by a stream of nitrogen gas.

In order to promote bacterial adhesion onto defined spots on the surface, the passivated surfaces were patterned using μCP with one of three chemicals, PD, PLL or PEI, all characterized by their expected ability to promote bacterial adhesion. The chemicals were patterned using the following procedures: Dopamine hydrochloride (Sigma-Aldrich) was dissolved in TRIS buffer (Sigma-Aldrich, pH = 8.5) (final concentration equal to 1 mg/mL) in order to initiate the polymerisation into polydopamine. A drop of this solution was transferred to a PDMS stamp for incubation for 30 minutes. PLL: Poly-L-lysine (Mw 15.000–30.000, FITC Labeled, Sigma-Aldrich) was dissolved in MilliQ water to a consentration of 1mg/mL and incubated on a PDMS stamp for 10 minutes. PEI: poly(ethyleneimine) (Mw 750,000 by LS, 50 wt % in H_2O, Sigma-Aldrich) was dilluted in MilliQ water to a 1% wt solution before incubation on a PDMS stamp for 10 minutes. After incubation the stamps were dried with a stream of nitrogen and the stamps were placed pattern side down on the glass bottomslides of Willco-dishes. After patterning of the glass bottom slides, the Willco-dishes where assembled following the manufacturers instructions.

Patterned surfaces with PD islands on PEGylated surfaces were imaged using Multimode V AFM (Digital Instruments/VEECO) equipped with J scanner operated in tapping mode under ambient conditions. Silicon nitride cantilevers PPP-NCH (Nanosensors, nominal resonant frequency 204–497 kHz and nominal spring constant 10–130 N/m) were used. Overlapping of trace and retrace signal was used as a prerequisite for adequate and high-quality image acquisition.

Bacterial strain, plasmid, growth media, and DNA transformation

In this study the *Pseudomonas putida* KT2440 (TOL plasmid cured derivative [33]) was utilized. *P. putida* KT2440 was grown in LB (10g/L tryptone; 5g/L yeast extract; 5g/L NaCl) supplemented with 50 μg/mL kanamycin at 30°C over-night in shake flasks. The plasmid pSB-M1g [34] was used to express the green florescent protein variant mut3 (GFP) from the

Pm promoter. This plasmid harbors the positively regulated XylS/Pm positive regulator/promoter system which can be induced by the passively diffusing 3-methylbenzoic acid (MB) (Sigma-Aldrich), a mini-RK2 replicon for vegetative replication, and a kanamycin gene as antibiotic resistance marker. Plasmid pSB-M1g was transferred into *P. putida* KT2440by electroporation [35].

Immobilization of bacteria onto μCP patterned glass surfaces

In order to obtain bacterial microarrays, the chemically patterned glass bottomed Willco-dishes obtained as described above, were incubated for 5 minutes with the over night grown *P. putida* KT2440 culture in LB medium. Once rinsed in distilled water in order to remove any unattached bacteria, LB was added to the dish to minimize the stress induced in the attached bacteria.

The viability of attached bacteria was investigated using a live/dead assay (LIVE/DEAD BacLight bacterial viability kit from Life Technologies AS). The live/dead assay was added to bacterial microarrays in Willco-dishes immediately after bacterial attachment to the arrays. When using the live/dead assay, bacteria with intact cell membranes are expected to emit green fluorescent light when illuminated with the appropriate excitation light, and these bacteria were considered alive. Bacteria with damaged cell membrane emit read fluorescent light, as a nucleic acid stain can reach the bacterial DNA, and these bacteria were considered dead.

As a proof of concept, the immobilized *P. putida* KT2440 harbouring the plasmid pSB-M1g, while on a microscope, was induced with MB. This was achieved by changing the liquid covering the bacteria from LB to LB containing 0.3 mM MB. The presence of the inducer initiates the expression of the GFP from the positively regulated XylS/Pm positive regulator/promoter system [34]. Upon induction the expression of GFP in the bacteria was followed using time laps imaging.

The bacterial arrays were inspected using a Leica SP5 confocal microscope.

Results and Discussion

When aiming at controlling bacterial adhesion, optimization of surface chemistry is essential. In the present study, in addition to clean glass, three different anti-adhesion coatings where investigated: BSA, PVA and PEG. These were investigated in combination with three chemicals commonly used to promote bacterial adhesion: PD, PLL and PEI. The twelve resulting combinations were all evaluated in order to identify the optimal combination for selective bacterial adhesion onto predefined surface areas. For these investigations PDMS stamps with lines of 5 μm width where used (Fig 1d). After incubation with bacterial suspensions containing the bacteria *P. putida* KT2440, the patterned surfaces were covered in LB and imaged using light microscopy (Fig 2). The result revealed that cleaned glass surfaces did not to a sufficient extent reduce bacterial adherence (Fig 2), emphasizing a need for a passivating surface coating. The density of bacteria adhering to the BSA coated surface areas was similar to that observed for the uncoated glass. BSA thus does not meet the criteria defined for an anti-adhesion layer. When the bacterial arrays obtained on PVA-coated glass surfaces were covered with LB medium the PVA coating showed a unsatisfying tendency for bacterial attachment, similar to BSA and clean glass. However, when these arrays were dried immediately after the bacterial incubation step, clearly defined lines of adhered bacteria where obtained (data not shown). This indicates that PVA does have a potential as an anti-adhesion coating, but its successful use requires further optimization of the process. An additional challenge related to the PVA film was its tendency to peel off of the glass substrate, sometimes within less than one hour after being deposited and then thermally cured to the glass surface. Based on these limitations PVA was not

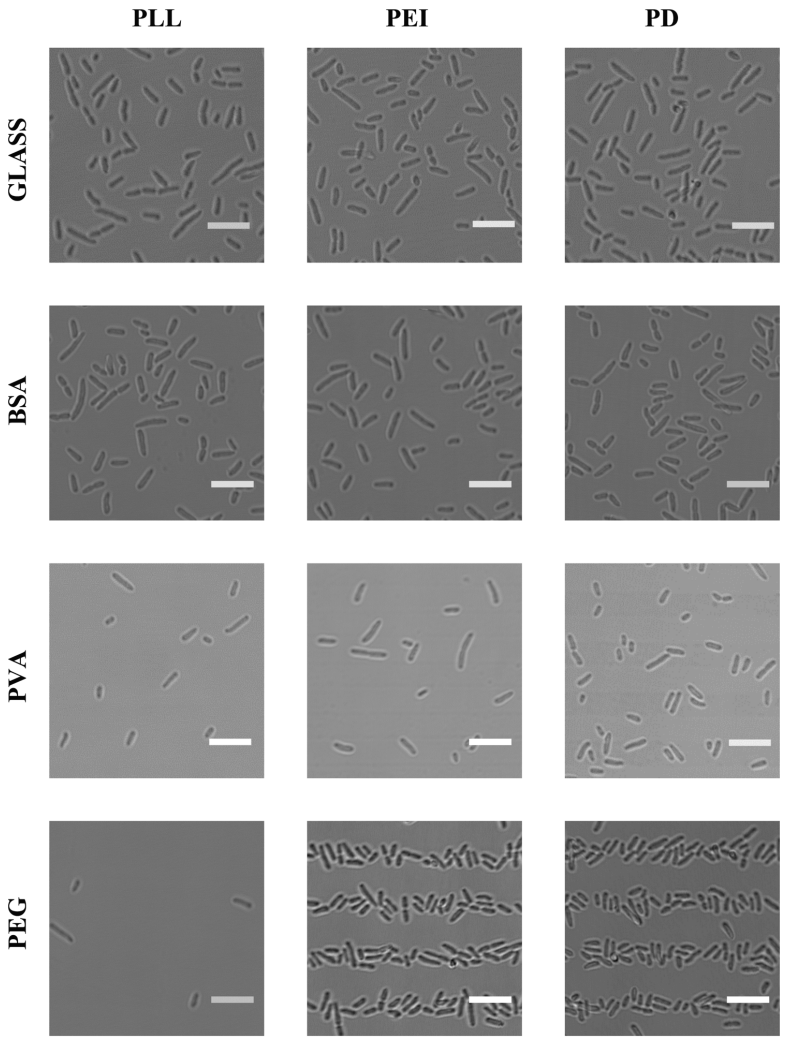

Fig 2. Images of glass surfaces and glass surfaces precoated with chemicals reducing bacterial adhesion, patterned with chemicals promoting bacterial adhesion, immersed in a solution containing bacteria and finally rinsed and covered with LB. Results obtained for the three chemicals potentially reducing bacterial adhesion (BSA, PVA or PEG) are shown. The substrates are patterned with one of three chemicals promoting bacterial adhesion (PLL, PEI or PD) using μCP with a PDMS stamp with 5 μm lines (Fig 1d) and immersed in a solution containing bacteria. All scalebars are 10 μm. The combination of chemicals investigated in each experiment is indicated on the figure. The surfaces were rinsed in MilliQ water after the incubation with bacteria (*P. putida* KT2440) in order to remove weakly adhering bacteria. During imaging the surfaces were covered with LB in order to minimize stress induced in the attached bacteria. The images are obtained by using transmission light microscopy, and were captured on a Leica TCS SP5 with a 40 × objective (water, N.A. = 1.2).

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used as an anti-adhesion coating in this study. PEG, on the other hand, efficiently prevent bacterial adhesion to areas in between the patterned bacterial adhering chemicals PEI and PD (Fig 2).

Of the three bacterial adhesion promoting chemicals tested, only PEI and PD produce well defined patterns of adhered bacteria on PEG films (Fig 2). The lack of patterns of adhered bacteria on surfaces patterned with PLL is thought to be the result of the PLL dissolving in the liquid covering the patterned surfaces as the patterns of FITC-labeled PLL could not be observed using a fluorescence microscope. The bacterial arrays are intended to be used for study of immobilised bacteria covered in LB to minimize stress, and PLL was thus not included in the further studies. No observed difference in suitability between PEI and PD was observed on striped patterns. However, for patterns with smaller feature sizes, deposition of PD resulted in an improved tendency for immobilisation of bacteria relative to PEI (data not shown). Patterns of PD on PEGylated surfaces were therefore chosen for the further investigations.

Immobilization of single bacteria onto adhesive spots on a patterned surface does not only require optimization of the surface chemistry, pattern features like spot size and inter-spot distance must also be optimized. To this end two different photolithography masks were designed and used to obtain PDMS stamps presenting pillars of varying diameter and separated by varying inter pillar spacing (Fig 1). The design presented in Fig 1a on the left side was inspired by a previously published design used for immobilizing *E. coli* [16] and consisted of 13 circular holes of increasing diameter on an opaque background. 11 out of the 13 circular features in the designed pattern on the first mask were successfully reproduced in the PDMS stamps (Fig 1b). The results obtained based on this mask, guided the design of a second mask. The pattern on the second mask consisted of circular holes with a diameter of 3.5 μm with a separation distance d_3 between the circular holes equal to either 10 or 15 μm (Fig 1a, right side). This pattern was successfully reproduced in the PDMS stamps (Fig 1c).

The PDMS stamps were used to deposit chemicals on glass surfaces. In order to evaluate the the successfulness of the deposition over relatively large areas (up to 9 mm^2), surfaces patterned using PDMS stamps coated with quantum dots were used (Fig 3, right). The patterns obtained

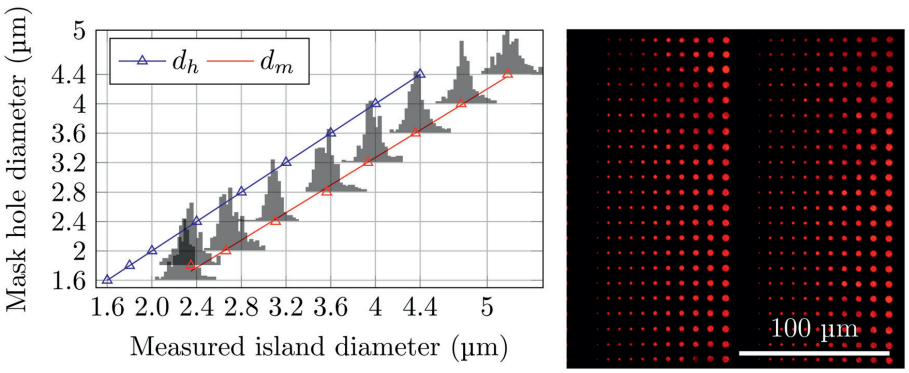

Fig 3. Right: Fluorescence micrograph of quantum dots deposited on a cleaned glass coverslip using μCP with PDMS stamps. Such images were used to study the reproducibility of the obtained patterns. Left: Distributions of observed diameters of the nine largest stamped islands compared to the mask hole diameters (blue triangles and corresponding blue linear regression line). Island diameters calculated from the area of each island as determined based on the ImageJ software and fluorescence micrographs of quantum dots. The red triangle indicate the most probable island diameter d_m and the red line is the linear regression obtained based on d_m obtained for the eight largest stamped islands.

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were reproducible over large areas (data not shown). Image analysis revealed a narrow distribution of island sizes (Fig 3, left). The sizes and size distributions of the islands of deposited quantum dots were found to be independent of the precise area of the stamp used to produce the printed features. The variation observed between different stamps produced using the same photolithography mask and identical parameter settings during stamp production was also insignificant. Furthermore, the size of the islands were compared with the size of the holes in the photolithography mask used when preparing the PDMS stamp. The most probable measured diameter (Fig 3, red triangles), defined as the peaks of the histograms presented in Fig 3 were compared to the designed diameter on the photolithography mask (Fig 3, blue triangles). The deposited islands were found to be larger than the holes in the photolithography mask (Fig 3, left). This is a systematic effect caused by the photolithography process and it can be tuned by adjusting the exposure dose. The patterns of stamped PD on PEGylated glass matches both the stamp features and the patterns of deposited quantum dots, as confirmed by AFM imaging of an array of PD on PEGylated glass (Fig 4).

Having identified PD and PEG as an effective combination of bacterial promoting and preventing chemicals, PD were μCP onto PEG coated surfaces using PDMS stamps with pilars of increasing diameter (Fig 1b). The obtained patterned surfaces gave bacterial arrays which successfully reproduced the pattern on the stamp. The preparation of single bacterial arrays requires that the bacterial adhering spots have a size that is sufficiently large to allow stable attachment of one bacterium, yet sufficiently small to minimize the probability for adherence of multiple bacteria. The number of bacteria immobilized on each spot of the arrays was determined by manual inspection of dry arrays for increased contrast in the images and revealed a correlation between the spot size and the number of bacteria adhering to the spot. Fig 5

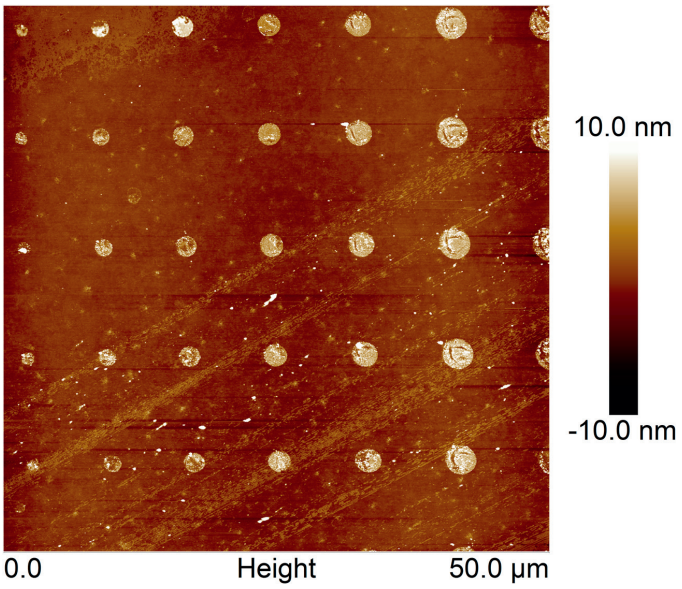

Fig 4. Tapping mode AFM height topographs of PD printed on PEGylated glass.

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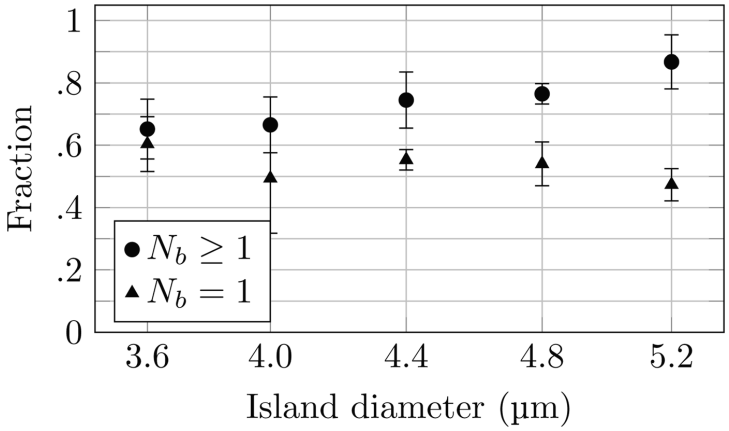

Fig 5. Quantitative analysis of the number of *P. putida* KT2440 adhering onto each of the PD islands on the obtained microarrays. For each PD island size, both the fraction of the spots having one or more bacteria attached ($N_b \geq 1$) as well as the fraction of the spots with only one bacterium attached ($N_b = 1$), were determined.

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displays this analysis for deposited spots with a measured size in the range 3.6 to 5.2 μm. Guided by these observations and the documented relationship between mask hole diameter and measured island size (Fig 3) a photolithography mask with holes of a size equal to 3.5 μm was chosen for the further studies. This is a compromise between a high probability of obtaining full coverage of the array, which is obtained for spot sizes large enough to capture several bacteria, and obtaining single bacterial arrays, which requires a spot size so small that a relatively large fraction of the spots remains unoccupied after incubation. The 3.5 μm spot size should give a large degree of coverage, while still keeping the average number of bacteria on each spot small enough for data analysis to recognize single bacteria. In addition to the spot sizes, the inter-spot distances were also evaluated. The arrays obtained revealed that even for separation distances equal to 8 μm, i.e. the largest distance included in the photolithography mask (Fig 1a), a fraction of the spots were bridged by the bacteria. This was especially apparent for the larger spots. Based on these findings, the pattern for a second photolithography mask was designed. The pattern had the following characteristics: holes of 3.5 μm diameter separated by either 10 or 15 μm (Fig 1c).

PD coated PDMS stamps prepared using the second photolithography mask allowed preparation of regular bacterial arrays on PEGylated glass surfaces. The number of bacteria immobilized on each adhesive island on the arrays were determined (Table 1). The inspection of five parallel μCP PD arrays on PEG coated surfaces revealed that the fraction of spots occupied by one or more bacteria was between 97 and 100% whereas the fraction of spots with single bacterial occupancy varied from 21.4 to 62.2% (Table 1). The amount of bacterial adhesion to the PEG coated areas was insignificant, as was the fraction of spots bridged by bacteria. The proposed method for the making of bacterial microarrays has several advantages compared to previously proposed methods, in the sense that it does not require modification of the bacteria and the surface modification procedure is fast and does not involve harmful chemicals. The size of the islands obtained in the current study, being approximately 10 μm^2, is also significantly

Table 1. Quantitative analysis of the number of bacteria immobilized onto each adhesive PD island of bacterial microarrays prepared on glass surfaces coated with PEG.

Array number	Number of islands	$N_b \geq 1$	$N_b \geq 1$ (%)	$N_b = 1$	$N_b = 1$ (%)
1	961	958	99.7	370	38.5
2	1972	1952	100.0	744	62.2
3	1764	1725	97.8	532	30.2
4	1444	1407	97.4	379	26.3
5	576	560	97.2	123	21.4

The arrays were prepared using μCP with PDMS stamps obtained using a photolithography mask with d_h equal to 3.5 μm (Fig 1d). $N_b \geq 1$: one or more bacteria per island. $N_b = 1$: one bacterium per island.

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smaller than the sizes used in other recently published procedures [23]. This small island size explains the low number of bacteria attached to each functionalized surface spot.

The observed variation in the fraction of spots displaying single attached bacteria (Table 1) might be due to variations in the feature sizes in the PDMS stamps. A small increase in the diameter of the PD islands will lead to an increased probability of adherence of multiple bacteria to each spot. The exposure dose during the photolithography process for making the mold, the amount of PDMS shrinkage during curing and the pressure applied during PDMS surface stamping are parameters that might influence the feature size of the stamped pattern and further optimization of these steps are therefore likely to further increase the probability for single bacterial occupancy. In addition, relatively large separation distance between the pillars of the PDMS stamp used may complicate the reproducibility of the stamping process.

The viability of bacteria attached to PD patterns on PEGylated surfaces was investigated using a live/dead viability kit. Bacteria with intact cell membranes are stained green and considered alive, whereas bacteria with damaged cell membrane are stained read as a nucleic acid stain can reach the bacteria DNA and are thereby considered dead. The live/dead assays revealed that the majority of the bacteria were viable while being immobilized onto patterned substrates (Fig 6). Out of a total of 3101 attached bacteria, 99.1% where stained green.

Arrays of *P. putida* KT2440 were exposed to MB, leading to expression of GFP from the positively regulated XylS/Pm system. Upon induction the expression was followed by microscopy. The introduction of MB was achieved by exchanging the medium covering the bacteria with LB containing 0.3 mM of MB. Bright field and fluorescent images of the bacteria were captured every ten minutes after adding the inducer (Fig 7). A green fluorescent signal was observed from the bacteria within an hour after adding the inducer. The observed fluorescence intensity increased over time. This time laps imaging also revealed that the bacteria were dividing while being immobilized on the array (Fig 7). These observations, along with the live/dead assay, show that the bacteria not only survive the immobilization process, but also that any stress induced by the immobilization does not significantly affect their growth. A variation in fluorescence intensity was observed between individual bacteria, and time from introduction of the inducer to the expression of GFP also varied between bacteria. This is an example of observed stochastic gene expression that leads to population heterogeneity. Such heterogeneity is masked in studies performed using methods that provide insight into average properties of bacterial populations.

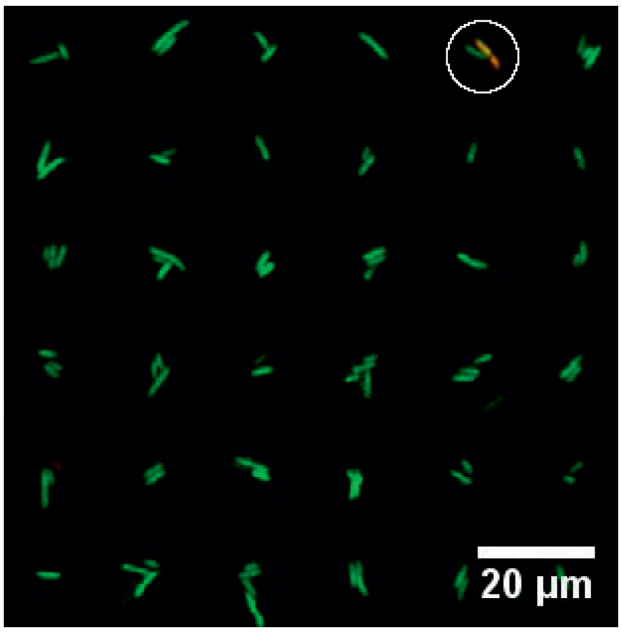

Fig 6. Fluorescence image reflecting the viability of *P. putida* KT2440 immobilized on arrays of PD islands on a PEGylated glass surface. Live bacteria are stained green, dead bacteria are stained red and the image is an overlay of both green and red fluorescent images. A single dead (red) bacteria is observed (white circle). The image is obtained for arrays covered in liquid using a Leica SP5 with a 10 × objective (N.A. = 0.4).

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Conclusion

Several conditions must be satisfied for a bacterial array to be an effective tool to study bacterial populations. The time and cost of making the array should be minimized, and the techniques used should preferably be applicable in a standard molecular biology laboratory. The chosen substrate should be transparent to allow for simple detection using optical imaging techniques while the bacteria are covered with liquid medium. In addition, the patterning technique chosen should not adversely affect the immobilized bacteria and the immobilization method should not require surface modification of the bacteria.

The present paper proposes a procedure for preparing microarrays of live bacteria that meets such criteria. In the proposed procedure, the substrates are patterned using μCP. Different combinations of chemicals for surface functionalization were evaluated. More precisely, the commonly used passivating chemicals PEG, PVA and BSA were tested in combination with the bacterial adhering chemicals PEI, PLL and PD. PEG-coated glass slides with printed PD patterns were shown to be effective at selectively immobilizing bacteria onto predefined areas on the surface. The design features of the PDMS stamps, including the diameter of each pillar on the stamp and the distance separating them, allowed the preparation of arrays of the bacteria *P. putida* KT2440 displaying high regularity, as reflected by the fraction of spots occupied by one or a few bacteria ranging from 97.2 to 100%. The proposed method for the preparation

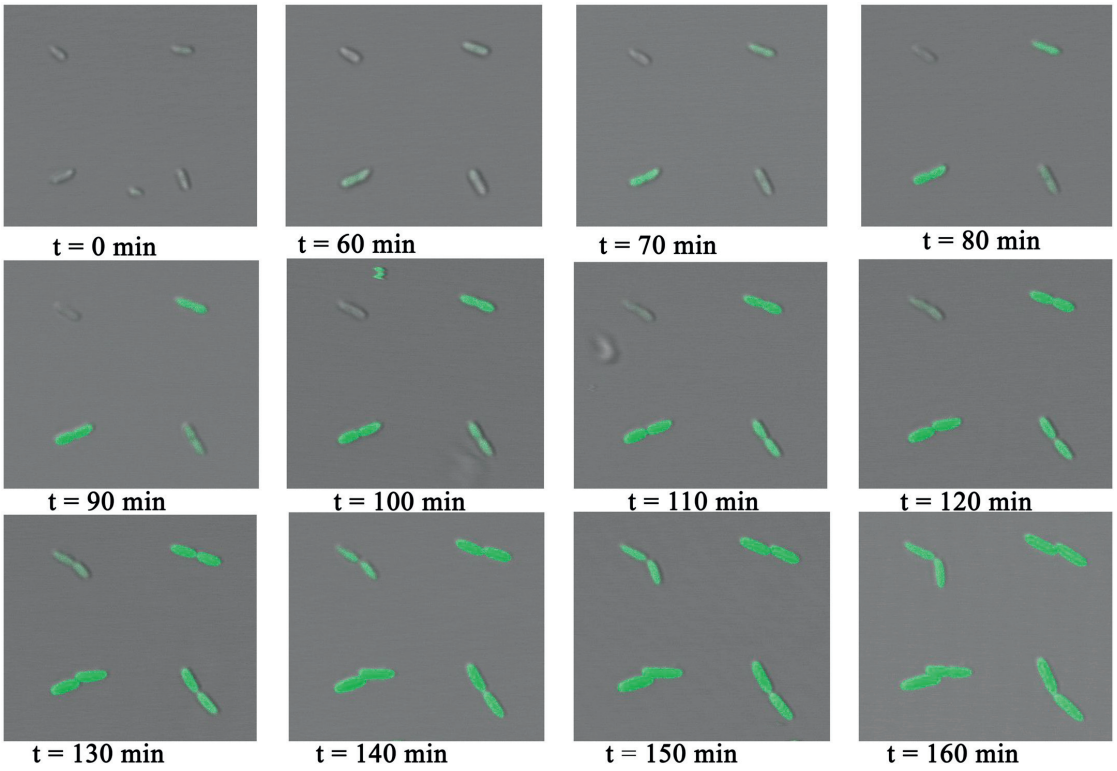

Fig 7. Time laps images of *P. putida* KT2440 immobilized on PD islands printed on a PEGylated glass surface. The images are obtained for surfaces covered with medium between 0 and 160 minutes after changing the medium from LB to LB containing the inducer MB. MB induces the expression of GFP in the bacteria. The images are overlays of transmission light images and fluorescence images—both obtained using a Leica SP5 with a 20 × objective (N.A. = 0.7).

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of bacterial arrays can be applied to any microorganisms for which a surface coating is identified that gives a high probability for attachment of the microorganism to the islands as well as a surface coating that gives a low probability for attachment to areas outside the islands. However, in the current study optimalisation of the surface coating for bacteria other than *P. putida* KT2440 was not performed. Furthermore, a live/dead assay revealed that 99.1% of the bacteria were alive after immobilization onto the array and bacteria attached to these arrays both divide and express GFP upon induction. The presently developed microarray with a large selectivity for single bacterial adherence to polydopamine μCP domains, and maintaining bacterial viability can be expected to support studies addressing bacterial heterogeneity.

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Author Contributions

Conceived and designed the experiments: NBA VO MS. Performed the experiments: NBA VO. Analyzed the data: NBA VO. Contributed reagents/materials/analysis tools: RL. Wrote the paper: NBA MS.

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Paper II

Journal Name

ARTICLE

Heterogeneity in GFP expression in isogenic populations of *P. putida* KT2440 investigated using flow cytometry and bacterial microarrays

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Individual bacteria, even bacteria belonging to an isogenic population under uniform environmental conditions, display phenotypic diversity and variability in gene expression. The increased focus that is lately given to this topic within microbiology and quantitative biology has motivated a quest for new experimental approaches providing insight into phenotypic traits at the single-cell level. In the present paper we investigate the applicability of bacterial microarrays for studies of bacterial populations including their inherent heterogeneity. Results obtained from microscopy of bacterial microarrays are compared to results obtained using flow cytometry. Two different positive-regulated promoter systems were utilized in isogenic cultures of *Pseudomonas putida*, both systems leading to green fluorescent protein production upon induction. Using both experimental approaches, a shift to higher fluorescence intensities with increasing time and increasing inducer concentration was observed, as well as a broadening of the distributions. Additionally, the micrographs of the bacterial microarrays revealed heritable inter-cell variation in green fluorescent protein levels. These observations indicate that for the bacteria studied here the cell to cell heterogeneity in green fluorescent protein expression, as also detected in the flow cytometer, are due to relatively static inter-cell differences.

Introduction

Microbial populations are traditionally studied at the population level, and the parameters determined reflect their average properties. With the development of experimental techniques that enable determination of many of these properties at the single-cell level, the heterogeneity of such populations has been revealed [1, 2]. Today it is therefore well accepted that individual bacteria, even bacteria belonging to an isogenic population under uniform environmental conditions, display phenotypic diversity and variability in their gene expression profile [3-7]. This phenomenon is often referred to as phenotypic heterogeneity, and was recently reviewed [8]. Gaining insight into this heterogeneity is recognized as important for further progress in the understanding of several aspects of the behavior of microbial populations, including stress response, adaptation and robustness towards changing environments [2].

The awareness about the importance of heterogeneity influences the choice of experimental strategies for the study of bacterial populations. When using population-based methods, the phenotypic variability is averaged out [9, 10]. The awareness of this limitation has motivated a quest for new experimental approaches that provide the ability to monitor phenotypic traits at the single-cell level. Phenotypic traits of cells and the distribution of these within a population are generally determined by gene sequencing, mass spectrometry and optical methods such as flow cytometry (FC) or microscopy [11].The combination of single-cell studies with genetically encoded fluorescent proteins that bind to defined structures within or at the surface of the cell has enabled monitoring of cellular properties, beyond size and shape, including protein localization and concentration[12]. In flow cytometers data are obtained on large numbers of cells that are observed one by one as they pass the laser spot of the FC instrument, in a time and labor efficient manner. Furthermore, FCs are widespread and accessible instruments, and FC has therefore been extensively used to gain insight into heterogeneity in biological systems [3, 5, 7] microbial population segregation [13, 14] as well as their response to antibacterial agents[15]. Whilst most flow cytometers have the advantages described above, some instruments have the additional ability to sort cells according to pre-defined phenotypic traits[1, 16, 17]. FC analysis can thus be complemented with further downstream

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investigation, such as microscopy, cultivation in liquid or agar plates, or even Omics-based technologies [11, 18-21].

In addition to the many fascinating studies based on FC, significant advances in the understanding of microbial phenotypic heterogeneity have been achieved using single-cell microscopy [22, 23]. The main advantage of this approach compared to FC is its ability to follow individual cells over time, and thus provide insight into the dynamics of cellular behavior. For example, bacterial cell division as well as the production of fluorescent gene-products has been studied at the single-cell level by tracking individual bacteria over prolonged periods of time [4, 22-24]. In some studies, it has been advantageous to separate the cells contained in a cell suspension prior to microscopy studies. Various physical principles have been exploited in microfluidic cultivation devices to trap, isolate and cultivate single-cells [25, 26]. However, 3D trapping and subsequent tracking of individual cells, especially in densely packed microcolonies, is demanding and sophisticated image recognition software is needed. The physical trapping of microorganisms in microfluidic devices can also, due to the closed system required for controlled liquid flow, lead to challenges in later steps aiming at the isolation of cells from the device for further investigations or cultivation.

Bacterial microarrays (BMs), where bacteria are immobilized onto spots on a surface, offer an alternative to the physical trapping in closed chambers in microfluidic devices. When using the microarray approach a high number of cells are studied simultaneously, and statistical information related to the distribution of phenotypic traits within a population can be obtained. Furthermore, microscopy of the BMs provide information on the behavior of the single-cells as a function of time, and the removal of cells from specific spots on the BM, as demonstrated by Iino *et al* who used a micro pipette to collect persister cells, opens for further investigations of cells belonging to interesting sub-populations [27].

We have previously proposed an approach for the preparation of bacterial microarrays (BMs) [28]. These BMs are obtained by immobilizing bacteria onto predefined spots on a glass surface by microcontact printing (μCP) of bioadhesive chemicals [28]. In the current paper we investigate the performance of the BMs for studying variation in phenotypes within isogenic bacterial populations. As part of the study results related to bacterial population heterogeneity obtained using the well-established FC approach are compared with results obtained based on microscopy of BMs. Two different positive-regulated promoter systems were utilized in isogenic cultures of *Pseudomonas putida*, both systems leading to green fluorescent protein (GFP) production upon induction. More precisely, the fluorescence intensity (F_{int}), due to induced GFP production from two positively regulated promoter systems, is quantified at the single-cell level, as a function of time after adding the inducer. The data obtained reveal cell-to-cell heterogeneity in these isogenic populations of bacteria.

Materials and Methods

***Pseudononas putida* strains and growth conditions**

Pseudomonas putida KT2440 (TOL plasmid cured derivative [29]) was grown in Lysogeny Broth (LB) (10g/L tryptone; 5g/L yeast extract; 5g/L NaCl) supplemented with 50 μg/mL kanamycin at 30°C over-night in shake flasks. Two different plasmids were utilized: pSB-B1b and a derivative of pSB-M1g, pHH100-GFP [30]. These plasmids provide the bacteria with the ability to express the GFP variant mut3 from the AraC/P_{BAD} and XylS/P_m positive regulator/promoter systems, respectively. The AraC/P_{BAD} system is induced by arabinose (Sigma-Aldrich), which requires an active uptake by the host cells, whereas the XylS/P_m system is induced by the passively diffusing 3-methylbenzoic acid (MB) (Sigma-Aldrich). Both plasmids are based on a mini-RK2 replicon with a kanamycin gene serving as antibiotic resistance marker. The plasmids were transferred into *P. putida* KT2440 by electroporation [31]. In this paper the cells carrying the plasmid pSB-B1b are referred to as strain A, and the cells carrying the plasmid pHH100-GFP are referred to as strain B.

Analysis of bacterial populations using flow cytometry

Flow cytometry measurements where performed using a Beckman Coulter Gallios (Beckman Coulter). For both strain A and strain B the GFP content of the individual bacteria (F_{int}) was quantified as a function of time after adding the relevant inducer (T_{ind}). Additionally, the inducer was added to four different final inducer concentrations (C_{ind}), studied in separate experimental series. The GFP was excited at 488 nm and the fluorescence detected in the wavelength interval from 505 to 540 nm, in accordance with the emission characteristics of GFP. Measurements were performed every 15 minutes for T_{ind} equal to 3 hours. The samples were prepared for flow cytometry experiments by transferring 6.6 μL of an overnight culture of *P. putida* to vials containing 3 mL LB with varying C_{ind}. For each C_{ind}, three parallel vials were prepared, and small aliquots were extracted and analyzed from each vial at different T_{ind}. Additionally, in each experimental series, a control sample containing bacteria but no inducer was investigated every 30 minutes. When quantifying GFP production in bacteria belonging to strain A the medium contained 1, 10, 25 or 50 mM of the inducer arabinose. When quantifying GFP production in bacteria belonging to strain B the medium contained 0.1, 0.25, 0.5 or 1 mM of the inducer MB. For each series, a total of 20,000 cells were counted. The data were processed using Kaluza (analysis software provided by Beckman Coulter). Based on the three parallel measurements performed for each C_{ind} and at each T_{ind}, the average F_{int} was determined.

Fabrication of bacterial microarrays

BMs of *P. putida* were prepared as previously described [28]. Briefly, the glass bottom (40 mm in diameter) of Willco dishes (WillCo Wells) were PEGylated by immersion in a solution containing 0.1 mg/mL PLL-g-PEG (Susos) for 60 minutes. The pegylated surfaces were then rinsed by immersion in phosphate buffered saline (Sigma Aldrich) followed by MilliQ water before being dryed using nitrogen

gas. A regular pattern of 4 µm in diameter spherical areas of polydopamine (PD) separated by 15 µm was deposited on the PEGylated surface. This was obtained by micro contact printing using a PDMS stamp pre-coated with PD through immersion in a solution containing PD (1 mg/mL in TRIS buffer at pH = 8.5, Sigma Aldrich). Immobilization of *P. putida* on these functionalized glass surfaces was obtained by leaving a drop of overnight culture on the surfaces for 5 minutes. Non-bound bacteria were removed by gentle rinsing with water, and the surfaces containing the arrays were immediately covered with LB.

Microscopy of bacterial microarrays

The BMs of *P. putida* were studied using a Leica SP5 confocal microscope. After positioning the BM on the sample stage of the microscope the LB medium covering the array was replaced with medium containing a predefined concentration of the inducer. The inducer concentrations were identical to those used in the FC experiments. The BMs were imaged every 15 minutes for a duration of three hours using a 20 X air objective (NA =0.7). Both bright field (BF) and fluorescence micrographs were recorded. Fluorescence was detected in the wavelength range 495 to 550 nm. The intensity of the laser (Argon-ion laser line 488 nm) and the detector settings were kept constant throughout the experiments. To compensate for drift of the sample in the z-direction and for potential tilt of the sample holder, causing uneven F_{int} across a micrograph, optical sections were acquired at five different focus positions. The spacing between each section of the z-stack was 500 nm and the center position in the z-stack was the focus determined using the autofocus function in the Leica software LAS AF.

Analysis of micrographs

The maximum intensity z-projection (MIP) function was applied to the z-stack of fluorescence micrographs, using the image processing software ImageJ (www.imagej.net). The corresponding z-stack of BF images was inspected and the image with the highest contrast between background and bacteria was stored, together with the MIP-image, for subsequent image analysis. MATLAB scripts were developed for image processing. In order to minimize effects of field curvature of the objective on the results, only bacteria located inside an inscribed circle of diameter 0.9 x *l* were analyzed, where *l* is the size of the original image (Fig 1A). All cells, including the non-fluorescent ones, were identified. The background of the images was determined by morphological opening on the BF-image using a spherical structuring element of radius 2 pixels. This background was subtracted from the original image one and the intensity and contrast subsequently adjusted (Fig 1B). To reduce noise, a median filter using a 3x3 neighborhood was applied. The resulting image was segmented by the Otsu thresholding method[32]. In order to separate spatially overlapping bacteria, dilated branch points were subtracted from the image. This binary mask (Fig 1C) was then used to localize the pixels of each cell in the fluorescence micrograph. The total fluorescence emitted from a cell was obtained by summarizing the gray values of the pixels of the individual cell.

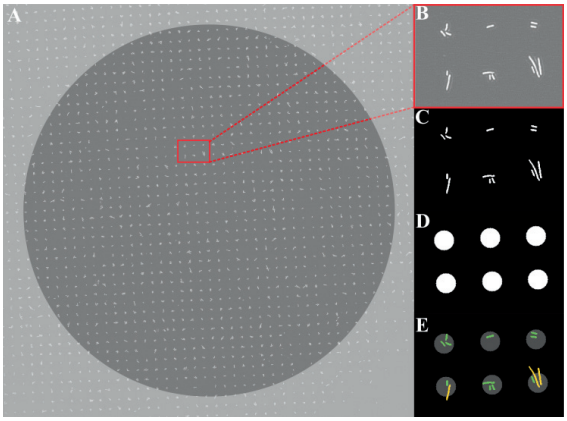

Figure 1: Key steps in the image analysis procedure applied to extract quantitative information from micrographs of BMs. A: Full-sized background-subtracted bright field image of the bacterial microarrays. For illustrative purposes, the image has been subjected to intensity and contrast enhancement. B: A magnified section of the image displayed in A. C: Binary image corresponding to the image section displayed in B. D: Binary image of the spots. E: Image resulting from the merging of image C and D. Bacteria localized inside the PD covered spots are colored green and bacteria localized outside or partly outside these spots are colored yellow.

A binary image containing information about the location of the PD spots (Fig 1D) was created as explained in the following. Since the spots were not visible in neither the BF-image nor the MIP-image, the center coordinates of each spot was approximated based on the fluorescence pattern in the MIP-image, assuming that the bacteria were randomly immobilized onto the PD-spots. Only bacteria localized completely or partly within the spots were included in the subsequent analysis. A radon transform was applied to the fluorescence pattern, to identify the two angles of orientation of the PD-spots. The local fluorescence maxima along the parallel and perpendicular lines at these angles corresponded to the center coordinates of the spots. The interval between the local maxima on each line was approximately 15 µm. With the center coordinates and the radius of each PD-spot determined, the binary image of the microsized pattern could be computed (Fig 1E) and the bacteria classified based on their location relative to a spot.

Results:

Characteristics of the bacterial microarrays

Figure 2 presents representative bright field micrographs obtained for BMs of *P. putida* belonging to strain B. The micrographs were analyzed using a custom-made MATLAB script that allowed extraction of quantitative statistical data. Table 1 presents the results obtained based on micrographs obtained 10 minutes after adding the inducer (T_{ind}=10) for six different BMs. In this analysis a total of 7293 spots were detected, of which 900 were empty (Table 1). The percentage of empty spots varied from 2.1 % to 15.9 %. The average percentage of empty spots was 12.3% and a total of 14,906 bacteria where counted. 10.9% of the detected bacteria were located outside the PD spots while 89.1 % of the bacteria were either inside or partly inside a spot. When excluding the empty spots from the analysis, the average number of bacteria per spot was 2.3.

Table 1: Number and localization of bacteria on PD spots*.

Strain	C_{ind}	Total number of features			Location of bacteria relative to disks		
	mM	Spots	Empty spots	Bacteria	On spots	On borders	Outside
A	50	1250	124	3105	1439	1035	631
	25	1214	189	2397	1740	466	191
	10	1221	102	2431	1805	355	271
	1	1198	145	2521	1705	612	204
B	1	1211	25	2697	2381	356	23
	0.5	1199	315	1755	1121	332	302
SUM		7293	900	14906	10128	3156	1622

The analysis is based on the first images obtained of the arrays after adding the inducer. These images were obtained at T_{ind} = 5 to 30 minutes.

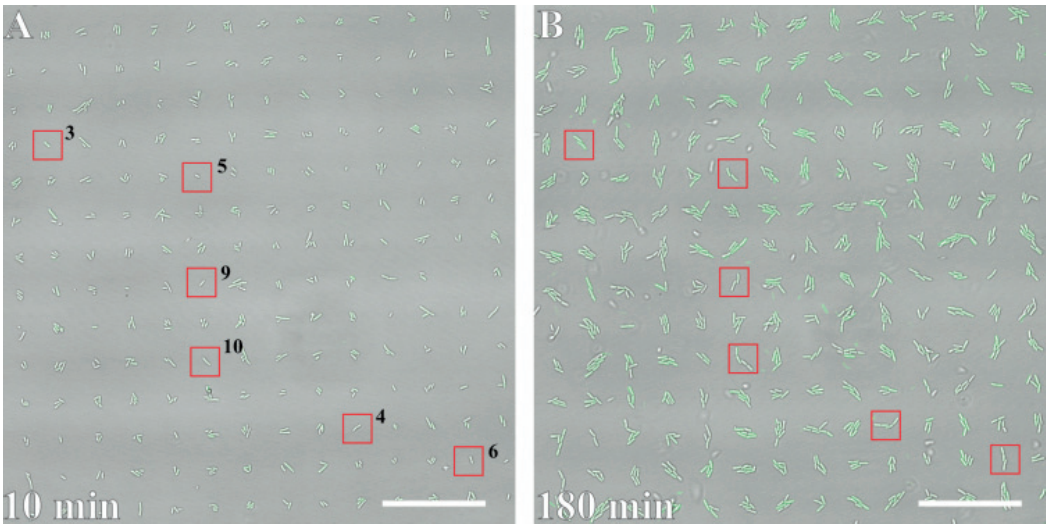

Figure 2: Bright field micrographs of BMs of *P.putida* belonging to strain B. The images were obtained 10 min (A) and 180 min (B) after adding the inducer MB at a concentration C_{ind} equal to 1mM. Scale bar =50 µm. The squares and numbers overlaid on the micrographs identify bacteria that were subjected to the analysis presented in figure 7.

Comparison of results obtained using FC and BMs

F_{int} was determined at the single-cell level as a function of C_{ind} using both FC and the array approach (Fig 3). The points in the graphs reflect the average F_{int} per bacterium determined at different T_{ind}. When using FC this was determined based on an average of three parallel measurements, as described in the materials and methods section. For the BMs the average fluorescence per cell was calculated by summarizing the fluorescence detected for each pixel element contained in the image of the cell. For the bacteria belonging to strain A, the two lowest C_{ind} give rise to only small changes in F_{int} with increasing time after addition of the inducer (Fig 3B). For the two highest C_{ind} investigated, 25 mM and 50 mM, F_{int} increases and reaches a plateau after approximately 100 minutes. This is visible in data obtained both using FC (Fig 3A) and BM (Fig 3B). For the bacteria belonging to strain B, the same tendency is observed: the two lowest C_{ind}, 0.1 mM and 0.5 mM, lead to only small increase in detected fluorescence (Fig 3C). Therefore, for strain B only the two highest concentrations were studied on the BMs (Fig 3D). Whereas the FC data obtained for strain B indicate a plateau in the fluorescence at approximately 160 minutes, this is not visible in the BM data (Fig 3D).

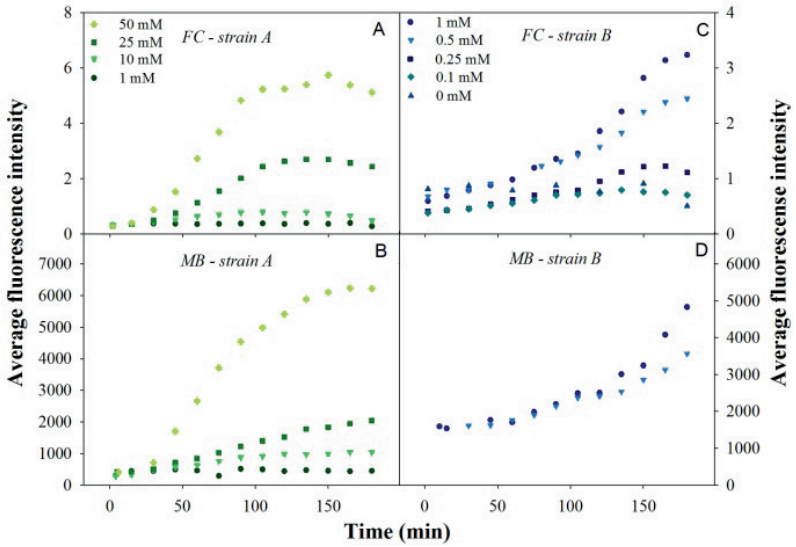

Figure 3: Average fluorescence emitted per bacterium presented as a function of time after adding the inducer. The concentrations displayed in A and C refer to C_{ind} of the inducers arabinose and MB, respectively.

In figure 4 the distribution of average fluorescence intensities are presented for strain A as a function of T_{ind} for three different C_{ind}. The analysis of both FC and BM data reveal a shift to higher fluorescence intensities as well as a broadening of the distribution with increasing T_{ind} and increasing C_{ind}. The average number of cells identified on the BMs underlying the analysis presented in figure 4 were for the examples shown 3084 ± 842 (C_{ind} = 10 mM), 3008 ± 648 (C_{ind} = 25 mM) and 3822 ±975 (C_{ind} = 50 mM). A similar analysis performed for strain B (Fig 5) reveal similar tendencies with respect to increase in average F_{int} and broadening of the intensity distribution. The average number of cells identified on the arrays were 2518 ± 892 (C_{ind} = 0.5 mM) and 3335 ±629 (C_{ind} = 1.0 mM).

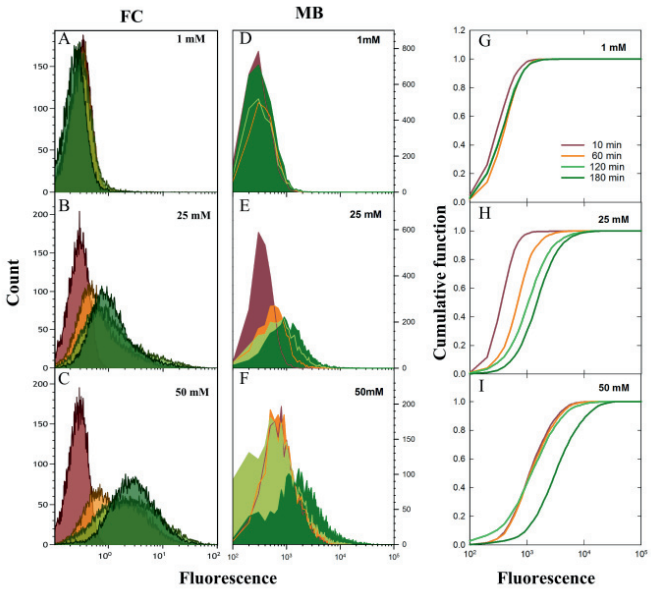

Figure 4: Distributions of F_{int} for bacteria belonging to strain A studied using FC and BMs. A-F: Histograms obtained for strain A based on data obtained using FC (A-C) and BMs (D-F). Data are shown for C_{ind} equal to 1 mM (A and D), 25 mM (B and E) and 50 mM (C and F) and at T_{ind} equal to 5 min (red), 60 min (orange), 120 min (light green), 180 min (dark green). G-I: Cumulative function of the distributions presented in D-F.

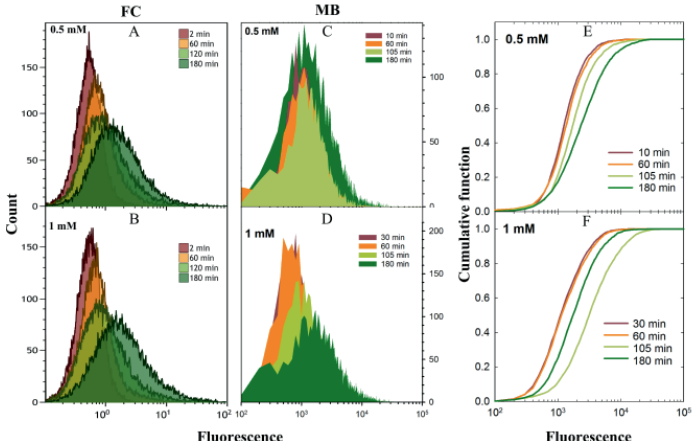

Figure 5: Distributions of F_{int} for bacteria belonging to strain B. A - D: Histograms obtained based on FC (A and B) and BMs (C and D) data. E - F: Cumulative function of the distributions presented in C and D. F_{int} was quantified for C_{ind} equal to 0.5 mM and 1 mM, and at T_{ind} equal to 5 min (red), 60 min (orange), 120 min (light green), 180 min (dark green).

Single-cell fluorescence intensity as a function of T_{ind} and C_{ind}

Microscopy of BMs allowed studying F_{int} for single bacterial cells belonging to strain A or B as a function of T_{int} (Figure 6 and 7). The different rows in figure 6A and 7A show images of single bacteria belonging to strain A or B, respectively, acquired at T_{ind} equal to 15, 60, 120 and 180 minutes. Whereas some bacteria show low and unaltered fluorescence emission upon addition of the inducer (Fig 6B, panel 2), most of the bacteria show increased F_{int} with increasing T_{int} (Fig 6 and 7). However, the rate of the increase in the F_{int}, as well as the final fluorescence level, varies between the different bacteria. Furthermore, the daughter cells seem to inherit the phenological traits of their mother with respect to fluorescence emission (Fig 6B and 7B).

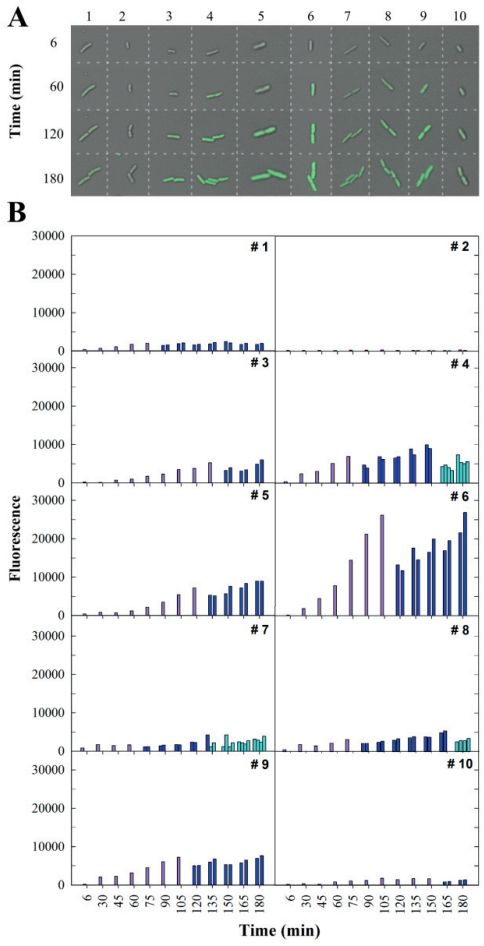

Figure 6: Increase in fluorescence emitted from bacteria belonging to strain A as a function of T_{ind} (C_{ind} = 50 mM). A: Magnified sections of fluorescence micrographs displaying 10 different bacteria and their daughter cells. The same cells are identified in micrographs acquired at T_{ind} equal to 15, 60, 120 and 180 minutes. B: Average F_{int} determined for the cells displayed in A as a function of increasing T_{ind}. F_{int} for the first, second and third generation of bacteria is shown in violet, blue and turquoise, respectively.

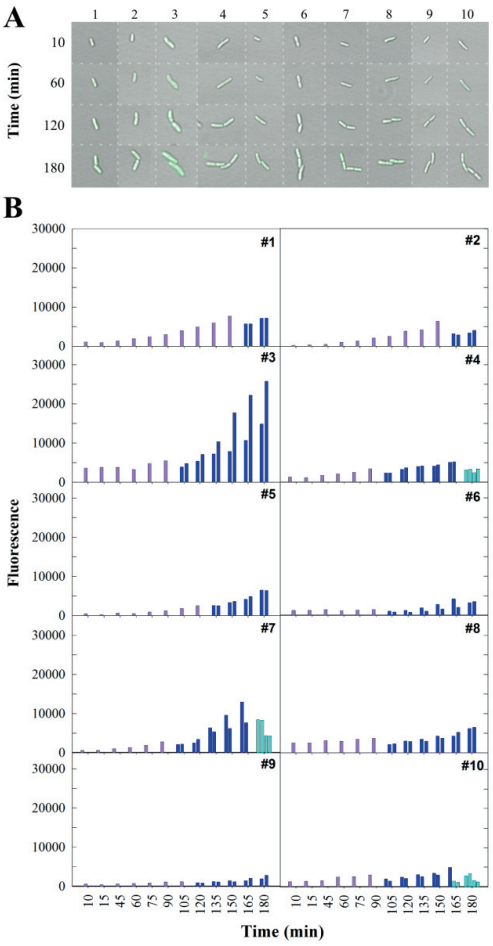

Figure 7: Increase in fluorescence emitted from bacteria belonging to strain B as a function of increasing T_{ind} (C_{ind} =1 mM). A: Magnified sections of fluorescence micrographs displaying 10 different bacteria and their daughter cells. The same cells are identified in micrographs acquired at T_{ind} equal to 15, 60, 120 and 180 minutes. B: Average F_{int} determined for the cells displayed in A as a function of increasing T_{ind}. F_{int} for the first, second and third generation of bacteria is shown in violet, blue and turquoise, respectively.

Upon cell division the size of the bacterium, and thus the total intensity of the emitted fluorescence, is reduced by approximately 50%, whereas the average fluorescence intensity per pixel element remains unchanged (Figure 8). Some of the bacteria studied divided twice during the three hours' time period they were studied (Fig 6 panel 4, 7 and 8, figure 7 panel 4 and 7). These observations indicate a generation time in the interval 75 to 90 minutes. The location of the bacteria presented in figure 6 with respect to the cumulative distributions of the bacteria (figure 4) reveal that the bacteria remain close to a defined percentile in the distributions (Figure 9).

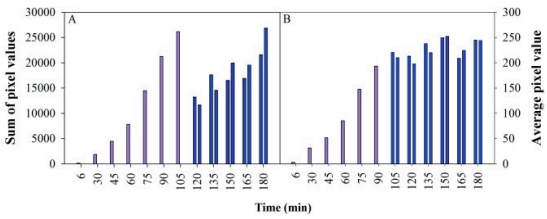

Figure 8: Increase in fluorescence intensity for a single bacterium as a function of increasing T_{ind}. A: F_{int} defined as the sum of the fluorescence determined in each of the pixel elements in the image of the bacterium. B: Fluorescence defined as the average value of the pixels elements in the image of the bacterium. The analysis is performed on the bacterium displayed in figure 6A #6.

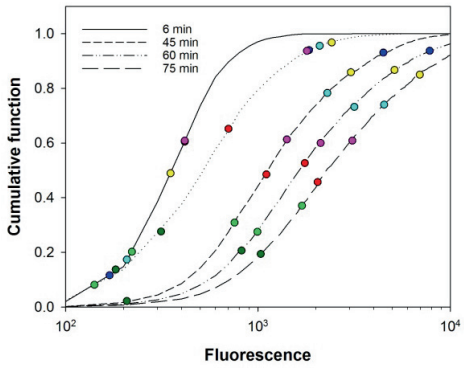

Figure 9: Cumulative function presenting the increase in F_{int} with increasing T_{ind}. The lines reflect the average F_{int} of a population of *P.putida* belonging to strain A. The symbols represent the fluorescence determined for some of the individual bacterial cells presented in figure 6. Among the cells presented in figure 6, the ones that divided prior to T_{ind} = 60 min or that showed very low and non-increasing F_{int} are not included.

Discussion

We have proposed an approach that makes imaging of high numbers of well separated single-cells possible in a non-labor-intensive way. The approach combines an advantage of FC, its ability to study high numbers of individual cells, with the advantages of fluorescence microscopy with respect to resolution and ability for time-lapse studies. The proposed method for the preparation of BMs gave arrays characterized by high regularity at short waiting times after immobilizing the bacteria (Fig 2 and table 1). The observed ability of the bacteria to divide on the arrays (Fig 2 right image) reflects the viability of the immobilized bacteria and is consistent with the reported results of a live/dead assay [28].

Fluorescence microscopy images often contain a vast amount of information, which is often hidden behind various sources of noise, convoluted with other information and stochastic in nature. Accessing the desired biological information can thus in many cases be facilitated by computational image analysis. The image analysis described in the current paper (Fig 1) illustrates how extraction of quantitative statistical data can be efficiently performed, and the BMs allow such analysis to be performed on a high number of cells. The automated analysis of high resolution micrographs of BMs is therefore a useful approach in studies aiming at revealing heterogeneity in bacterial behavior.

In order to validate the proposed approach, data obtained from the quantitative analysis of the BMs were compared to data obtained using FC (Fig 3). Similar increase in average F_{int} was observed using both approaches. The similarity of the data obtained using the two methods illustrate that the array approach provides correct information concerning the average behavior displayed by the populations. Good correspondence between the information obtained by the two techniques is also observed related to the distribution of average fluorescence intensities as determined on the single-cell level (Fig 4 and 5). Using both approaches, a shift to higher fluorescence intensities with increasing time and increasing C_{int} was observed, as well as a broadening of the distributions.

The fluorescence micrographs obtained for the BMs revealed that average F_{int} per cell as a function of T_{ind} vary significantly between the different bacteria. (Fig 6 and 7) For some of the bacteria F_{int} increased from 170 to 26140, a 15,000 % increase with time after addition of the inducer (Fig 6 #6). For other bacteria no increase in F_{int} was observed upon addition of the inducer (Fig 6 #2), and the remaining bacteria showed an increase in F_{int} intermittent of these

two extremes. The variation in F_{int} over time detected for a given bacterium was relatively small compared to the variation observed between the different bacteria (Fig 6 and 7). A significant fraction of the bacteria underwent cell division while being immobilized on the array. Cell division of bacteria immobilized to surfaces was also previously observed using AFM[33]. The fluorescence images reveal that the daughter cells show similar behavior as the mother cells with respect to F_{int} (Fig 6 and 7). F_{int}, reflecting the GFP concentration within the bacterium, thus appears to be a property that is inherited by the daughter cells. F_{int} deduced from the growth of the individual bacteria indicate that they follow the same percentile within the distribution (Fig 9). These observations indicate that for the bacteria studied here the cell to cell heterogeneity in GFP production, as also detected in the flow cytometer, are due to relatively static inter-cell differences.

Stochasticity in bacterial populations is known to result either from intrinsic or extrinsic sources. Intrinsic noise can be the result of stochastic promoter activation, promoter deactivation, and the rate of production and decay of mRNA and proteins, whereas extrinsic noise refers to the variation observed from external sources to the biochemical process and gene expression [4, 34]. In this study we have utilized two different positive regulated promoter systems, on the same replicon, which led to the observation of distinct heritable inter-cell variation in GFP levels in *P. putida* KT2440. What unifies both systems is the use of same replicon, the reporter gene, and the bacterial strain. The plasmid replicon utilized, mini-RK2 is a low-copy number plasmid with 5 to 8 copies per chromosome The mini-RK2 plasmid originates from the RK2 plasmid (60kb), but lacks the partitioning system which for intact RK2 leads to a plasmid localization inside the nucleoid in mid- or quarter-cell positions[35], ensuring the daughter cells receiving at least one copy of the plasmid The lack of this system leads to, in *E. coli*, localization of the mini-RK2 plasmid at the cell poles outside of the nucleoid as clusters [35] hence cell-to-cell variation in plasmid copy numbers. The localization characteristics of mini-RK2 replicon in *P. putida* are currently not known. However, if also this plasmid clusters at the cell poles during cell division this might account for the observed cell-to-cell variation (Fig 6 and 7).

The biochemical steps involved in production of GFP, as well as cell-to-cell variation in inducer uptake might be influenced by cell-specific factors which may also contribute to the observations described in figure 6 and 7. Regardless of the molecular explanation the variation in the observed phenotypic trait is a heritable one as the daughter cells formed exhibits the same level of GFP expression as the mother cell.

Conclusion

In this work, the applicability of bacterial microarrays for studies of heterogeneity in isogenic cultures of *Pseudomonas putida* was studied. A comparison of results obtained from microscopy of BMs with results obtained using FC allowed validating the BM approach. Using both experimental approaches, a shift to higher fluorescence intensities with increasing time and increasing inducer concentration was observed, as well as a broadening of the distributions. The two different promoter systems studied both led to the observation of distinct heritable inter-cell variation in GFP levels in *P. putida* KT2440. These observations indicate that for the bacteria studied here the cell to cell heterogeneity in GFP expression, as also detected in the flow cytometer, are due to relatively static inter-cell differences.

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Paper III

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