MICROFLUIDIC DEVICES FOR BIOTECHNOLOGY AND ORGANIC CHEMICAL APPLICATIONS

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The front cover is a montage of a scanning electron micrograph of streptavidin-coated beads self-assembled in a high-resolution pattern of lines as narrow as 5 µm.

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Abstract

Imagine if you could combine the power and capabilities of an entire laboratory in the palm of your hand. Advances in microfluidic chip technology promise to integrate and miniaturize multiple lab processes into a single palm-sized device. The advantages of these lab-on-a-chip devices, sometimes also referred to as micro total analysis systems (μTAS), compared with conventional bench-scale systems are numerous and wide ranging and include: less reagent consumption, low manufacturing costs, increased performance, faster analysis, high sample throughput, integration and automation possibilities, and disposability. However, microfluidic devices also present challenges such as the interfacing to the macro world and detection limits.

In this thesis the focus has been to develop novel discrete microfluidic components for biotechnology and organic chemical applications with the goal to integrate them to form lab-on-chips. A flow-through filter-chamber device has been designed, manufactured and evaluated for chemical analysis on beads. Passive liquid handling has been integrated on the chip in the form of hydrophobic valves at the inlet channels. An array format has also been developed to allow parallel analysis of multiple samples. The filter-chamber functions well for single nucleotide analysis using pyrosequencing. Initial evaluations on catalyst screening in the filter-chamber device has been performed.

The suitability of valve-less micropumps for biochemical applications is presented. Fluids encountered in various biochemical methods, including living cells, that are problematic for other micropumps have been pumped with good performance. This thesis also introduces expandable microspheres as a novel component in microfluidics including applications such as one-shot valves, micropositioning and surface enlargement.

A novel technique for bead immobilization in microfluidic devices based on surface chemistry is presented in this thesis. Beads for both biochemical assays and organic chemistry have been self-sorted and self-assembled in line patterns as narrow as 5 μm on both structured and unstructured substrates. This method will greatly facilitate the generation of screening platforms, for example.

To develop a microfluidic device for catalysis-on-chip, ligands for asymmetric catalysis have successfully been immobilized in silicon channels by consecutive microcontact printing, which is a novel technique presented in this thesis.

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ERRATA

Paper 1, line 6 in abstract, line 19 on page 206 and line 38 on page 207, 50 beads should be 5740 beads.

Paper 1, equation 1 is missing the parameter ‘L’ in the numerator.

Paper 3, line 16 on page 252, 50 beads should be 5740.

Paper 4, line 13 on page 263, ‘from’ should be omitted.

Paper 6, line 2 in abstract and line 17 on page 78, poly(methylsiloxane) should be poly(dimethylsiloxane).

In memory of Professor Börje Larsson
who first introduced me to the truest joys of
scientific research

Contents

List of Publications

The presented thesis is based on the following papers:

1. **Micromachined Flow-Through Filter-Chamber for Chemical Reactions on Beads**
   Helene Andersson, Wouter van der Wijngaart, Peter Enoksson, Göran Stemme
   Sensors and Actuators B: Chemical, 2000, 67, 203-208.

2. **Hydrophobic Valves of Plasma Deposited Octafluorocyclobutane in DRIE Channels**
   Helene Andersson, Wouter van der Wijngaart, Frank Niklaus, Patrick Griss, Göran Stemme
   Sensors & Actuators B: Chemical, 2001, 75, 136-141.

3. **Micromachined Filter-Chamber Array with Passive Valves for Biochemical Assays on Beads**
   Helene Andersson, Wouter van der Wijngaart, Göran Stemme
   Electrophoresis, 2001, 22, 249-257.

4. **Valve-Less Diffuser Micropump for Biochemical Microfluidic Applications**
   Helene Andersson, Wouter van der Wijngaart, Peter Nilsson, Peter Enoksson, Göran Stemme
   Sensors and Actuators B: Chemical, 2001, 72, 3, 259-265.

5. **Low Temperature Full Wafer Adhesive Bonding of Structured Wafers**
   Frank Niklaus, Helene Andersson, Peter Enoksson, Göran Stemme
   Sensors and Actuators, 2001, 92, 1-3, 235-241.

6. **Consecutive Microcontact Printing of Ligands for Asymmetric Catalysis in Silicon Channels**
   Helene Andersson, Christina Jönsson, Christina Moberg, Göran Stemme
   Sensors & Actuators B: Chemical, 2001, 79, 1, 78-84.

7. **Self-Assembled and Self-Sorted Array of Chemically Active beads for Analytical and Biochemical Screening**
   Helene Andersson, Christina Jönsson, Christina Moberg, Göran Stemme
   Talanta, In press.

8. **Patterned Self-Assembled Beads in Silicon Channels**
   Helene Andersson, Christina Jönsson, Christina Moberg, Göran Stemme
   Electrophoresis, 2001, 22, 3876-3882.

9. **SNP Analysis by Allele-Specific Extension in a Micromachined Filter-Chamber**
   Afshin Ahmadian, Aman Russom, Helene Andersson, Wouter van der Wijngaart, Mathias Uhlen, Göran Stemme, Peter Nilsson
   Submitted to BioTechniques

10. **Expandable Microspheres: A Novel Component in Microfluidics**
    Helene Andersson, Patrick Griss, Göran Stemme
    Submitted to Sensors & Actuators B: Chemical

The contributions of Helene Andersson to the different publications:

1 Parts of design, all fabrication and experiments. Major part of writing.

2 Parts of design, fabrication and all experiments. Major part of writing.

3 Parts of design, fabrication and experiments. Major part of writing.

4 No design, parts of fabrication, all experiments. Major part of writing.

5 Parts of fabrication, experiments and writing.

6 All device design and fabrication. Minor part of chemical design, parts of experiments and major part of writing.

7 All device design and fabrication. Parts of experiments and major part of writing.

8 All device design and fabrication. Parts of experiments and major part of writing.

9 All device design and fabrication. Minor parts of experiments and writing.

10 Parts of design, fabrication and experiments. Major part of writing.

1. Introduction

Over the past decade, miniaturization of analytical techniques has become a dominant trend in life sciences. The interest in this trend ranges, from laboratories interested in creating novel microfabricated structures to application laboratories focused on specific uses. Research into miniaturization is primarily driven by the need to minimize costs by reducing the consumption of expensive reagents and by increasing throughput and automation. The technology used to realize miniaturized systems was originally developed by the microelectronics industry. In the same way integrated circuits enabled for the miniaturization of computers from the size of a room to the size of a notebook, miniaturization has the potential to shrink a room full of instruments into a compact lab-on-a-chip.

In 1990 a new concept coined 'Micro Total Analysis Systems' was introduced and subsequently abbreviated to μTAS originating from the ideas of A. Manz, N. Graber and H. M. Widmer [1]. This concept proposed integrating the entire analytical process, from sampling through detection, into a microfluidic system, thereby enabling faster analysis. Just a few years later, the demonstration of capillary electrophoresis separations of picoliter samples in etched microchannels in glass chips established the validity of the μTAS concept [2]. The μTAS area has since then grown exponentially and evolved into a more general area of research encompassing different types of microfabricated chemical, biochemical and biological systems also popularly referred to as lab-on-a-chip. Applications of miniaturized systems in genetic analysis [3-5], clinical diagnostics [6-8], combinatorial chemistry [9-10], proteomics [11-12], drug discovery [13], and portable instrumentation [6-7] provide the drivers for this research and development.

The advantages of microfluidic devices compared with conventional bench-scale systems are numerous and wide ranging and include: less reagent consumption, low manufacturing costs, increased performance, faster analysis, high sample throughput, integration and automation possibilities, and disposability [12, 14]. However, microfluidic devices also present challenges such as handling of nanoscale volumes of liquids [15] and the interfacing to the macro world [16-18].

This thesis focuses on novel microfabrication techniques and microfluidic devices for applications in the fields of biotechnology and organic chemistry. To some extent this thesis reflects the diversity and interdisciplinarity involved when chemistry, biochemistry, and biology are combined with microtechnologies.

2. Objectives

The main objective of this work is to develop novel microfabrication techniques and microfluidic devices for applications in the fields of biotechnology and organic chemistry. The techniques should enable or facilitate the fabrication of microfluidic devices with certain specifications. This may involve the use of a conventional machine in an innovative manner or the combination of two or more conventional techniques into a new method with greater potential.

During the design phase of the microdevices the planned chemical applications influence the design. In that way the structures can be optimally tailored both in terms of the microfluidics and the chemistry. Two important criteria should be ensured in the devices designed within the context of this thesis research, simplicity and efficiency. The devices will be realized in silicon. However, since these devices are commonly considered for disposable use plastic replication might be considered after the functionality of the devices has been demonstrated in silicon. In addition, the devices must be easy to handle and allow the user to modify the reactive surface of the device outside the cleanroom.

3. Miniaturized Systems for Genomic Analysis

Genomics aims to characterize all the genes of an organism, including their sequences, polymorphism, structures, regulation, interaction and products. Genomics promises to revolutionize biological understanding, providing us with a molecular description of a biological entity and the effect of environment, disease and drugs upon it. To reach this goal, the scientists need tools that can deliver genetic information quickly, cheaply and accurately. The large amount of information within a single cell in terms of gene expression and proteins present requires that the technology gives results rapidly and affordably. Semiconductor microfabrication is an excellent platform for developing miniature integrated analysis systems with short analysis times, reduced sample-volume requirements and high cost efficiency. These microanalysis devices can be classified into two broad categories based on the complexity of the fluidics involved: surface microarray-based and microfluidic-based microdevices [12]. In the first case, arrays of minute spots of biological material are immobilized. The sample is then washed over the array and locations where samples have hybridized are analyzed, generally by fluorescence measurements.

In the microfluidic systems, material is transported within the microstructures where reactions, separations, and analysis can occur. These microfluidic systems are developments towards micro total analysis systems (μTAS) which perform the function of large analytical instruments but use much smaller devices [1]. Sampling, any type of pretreatment steps (such as filtration, reactions and separations) and detection will all be undertaken in the μTAS.

In this chapter some background and examples of both surface microarray-based and microfluidic-based microdevices will be given, including the new devices and techniques developed within the scope of this thesis. Advantages and disadvantages of the different systems will also be discussed.

3.1 Surface-Based Microarray Systems

The simplest form of a surface-based microarray system is drops of reagents or samples deposited directly onto a substrate. This is a solid-phase system with no surface enlargement at the area where the spots are positioned and there is nothing preventing cross-contamination between different spots, see a conceptual drawing in figure 1 (a). To allow isolation of analytes in picoliter volumes with low cross-contamination risk, small vials can be formed in the substrate, see figure 1 (b). Vials allow reactions and analysis to occur in free solution. To form a three-dimensional system with greater capacity for immobilization compared to a two-dimensional support (figure 1 (a)) gel pads can be immobilized on a substrate, see figure 1 (c). The gel pads create a porous and aqueous environment which resembles

homogeneous liquid-phase reaction conditions rather than heterogeneous solid-phase reaction conditions. In addition, each pad is separated by a hydrophobic film to avoid cross-contamination during the loading and analysis of the different gel pads. Bead arrays are another three-dimensional system where self-assembled beads constitute the solid-phase, see figure 1 (d). The immobilized beads are also separated by a hydrophobic film to enable parallel sampling handling.

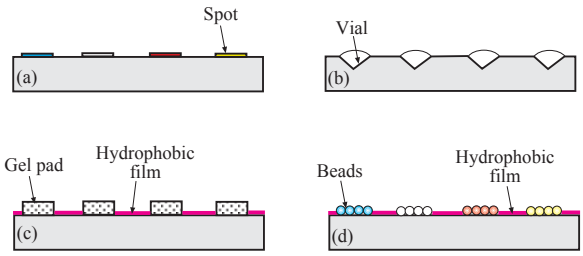


Figure 1. A conceptual drawing of different surface-based microarrays. In (a) drops of reagents or samples are deposited directly onto a substrate, in (b) small vials isolate picoliter volumes of analytes, in (c) three-dimensional gel pads are used for sample immobilization and in (d) immobilized beads constitute the solid-support.

In the following section the application of these four different surface-based microarray systems in genomic analysis will be described and compared with each other.

3.1.1 DNA Microarrays

In the past several years, a new technology called DNA microarray has attracted tremendous interest. Terminologies that have been used in the literature to describe this technology include, but not limited to, biochip, DNA chip, gene chip and genome chip. DNA microarrays allow scientists to analyze the presence, activity or sequence variations of thousands of genes simultaneously. DNA microarrays have been covered in the literature by a number of reviews [19-20]. Therefore, the aim of this section it not to provide a thorough review of these systems but to give a brief introduction to put the DNA microarrays into perspectives to other surface-based microarray systems.

A microarray starts with a piece of glass, silicon or sometimes plastics, the size of a microscope slide or smaller. Thousands of patches of single-stranded DNA (called probes) are fixed on this substrate, each spot measuring approximately 20-200 μm across depending on the immobilization method. The location and identity of each spot of DNA are known ahead of time. Microarrays rely on the so-called hybridization reaction. Two lengths of single-stranded DNA will bind together (hybridize) only if the bases on one strand find complementary bases on the other stand. The target, free DNA or RNA, is

labeled with fluorescent markers. It is washed over the array and hybridized to any single-stranded probe that has a complementary gene sequence to its own. A scan of the array with a laser or other excitation source causes any DNA that has found a tagged match to fluoresce. The light is collected by a detector, which can consist of a charge-coupled device (CCD) or a photomultiplier tube. The image is then fed to a computer that analyzes the location, color, and intensity of each patch of DNA. Because the identity of the array's DNA in each spot is known, the identity of any captured DNA is also known.

In figure 2 a scheme of the different steps involved in a comparative gene expression experiment is shown. Two sets of cells, for example normal and treated, provide the initial sample. The aim of the experiment is to investigate the gene expression in the cell populations by monitoring the messenger RNAs (mRNA) in the cell nucleus. In the first stage of the experiment, mRNAs are extracted from the cells and reverse-transcribed into more stable complementary DNA (cDNA). The cDNA from each cell population are then labeled, typically with different colored fluorescent dyes. The cDNAs are then hybridized to the DNA microarray. The hybridized array is then washed and scanned. The position and intensity of the spots seen provide information regarding the genes expressed by the cell. Note that while arrays are increasingly used for gene expression analysis, they only measure relative and not absolute levels of gene transcription.

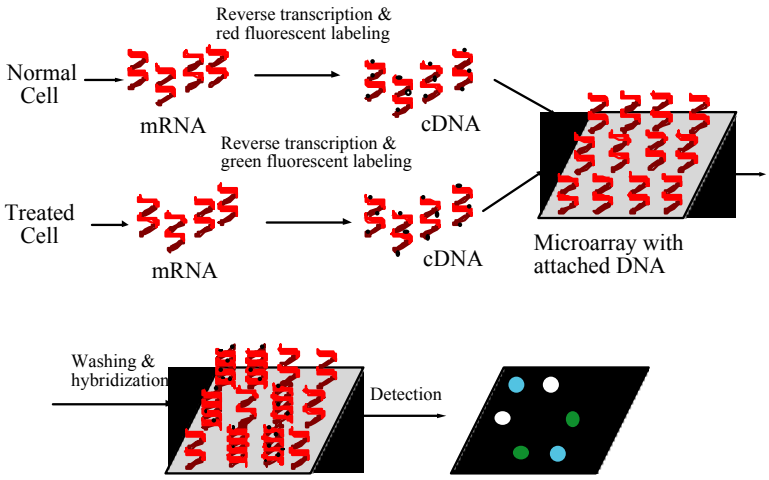


Figure 2. A schematic of a comparative gene expression experiment.

Microarray systems vary in the type of polynucleic acid immobilized, the methods of immobilization, nucleic acid formation (on chip or *ex situ*), hybridization and reading of the hybridized array. The

probes can take three different forms: DNA clones, PCR products or oligonucleotides, and may be immobilized onto the substrate by chemical or physical methods. The three main methods used for creating the arrays are pin-based fluid transfer [21], piezo-based inkjet dispensers [22], and photolithography [23]. A number of detection systems have been postulated and employed, ranging from radiochemical to electrochemical, but the most widely used is fluorescent labeling of the target DNA.

Affymetrix Inc. (Santa Clara, CA) is the current market leader in oligonucleotide microarrays and the owner of the commonly used term 'gene chips'. The Affymetrix GeneChips are high-density, miniaturized arrays of oligonucleotide probes on silicon support, see figure 3. Photolithography is used in this light-directed oligomer synthesis approach to chip production [23]. These chips have been successfully employed in a number of studies [24-26].

Figure 3. The Affymetrix GeneChip (http://www.affymetrix.com).

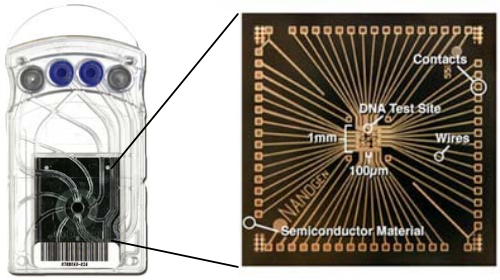


Figure 4. The NanoChip from Nanogen (http://www.nanogen.com/).

Nanogen Inc. (San Diego, CA) has developed a method of targeting DNA to specific sites on a silicon-based array using electronics [27], see figure 4. The system utilizes electric fields as an independent parameter to control transport, enhance hybridization and improve the stringency of the nucleic acid interaction. The system allows rapid hybridization, *i.e.* seconds compared to hours for a typical microarray system. Furthermore, by reversing the electric potential of the microelectrode, unhybridized molecules may be rapidly removed. Microarrays may use electric fields in other ways in the future.

There are now over 100 companies offering DNA microarrays, methods for producing arrays or systems for reading the chips. A list of these and a list of reviews of microarray systems can be found on Leming Shi's gene chip internet page, www.gene-chips.com.

In summary, DNA microarrays can have tens of thousands of probes to be used for identification of multiple targets with very high speed and high throughput. However, these systems do not have integrated microsensor detection systems, instead they usually have separate detection systems that are relatively large and are only suitable for laboratory-based research applications. Therefore, the development of microarrays with integrated optics has begun [28]. Since there is nothing preventing cross-contamination between the different spots, one chip is to be used for only one sample. One possibility to enable parallel sample analysis would be to integrate a microfluidic network which directs different samples to individual dots. However, the system then becomes much more complex in terms of fabrication and handling. To date, it is possible to analyze multiple samples on one array if the samples are labeled with different fluorophores [21]. Until today, mainly nucleic acid has been analyzed on the microarrays and since it is a heterogeneous solid-phase system, it may have limitations for analysis of proteins or other assays both in terms of immobilization and analysis.

3.1.2 Open Surface-Based Vials

Vials with nano- and picoliter volumes are basically small cavities etched or drilled in a substrate like silicon, glass or plastics, see figure 1 (b). The concept of miniaturized open chip-based nanovials was introduced by [29], where the nanovials were used as sample containers for electrokinetic injection of DNA samples prior to capillary electrophoresis separations. The usefulness of open vials, with nano- and picoliter sized volumes, has also been demonstrated as targets for matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of oligonucleotides [30], peptides [31], and proteins [32], and as reaction vessels for tryptic digests of myoglobin followed by an electrophoretic separation of the obtained peptide map [33-34], electrochemical studies [35], and bioluminescence measurements [36].

On an array of vials loaded with different samples the risk for cross-contamination is lower than if the different samples are deposited on an unstructured substrate, like in the DNA microarray case, due to the physical appearance of the vial. This could be further improved if the surface separating the vials is coated with a hydrophobic film. In the vials reactions take place in a homogeneous liquid-phase reaction rather than a heterogeneous solid-phase reaction, *e.g.* DNA microarrays, which might be a requirement for some chemical reactions.

The main difficulty in handling liquid samples in the nano- and picoliter regime is rapid evaporation. The rate of evaporation is proportional to the surface area to volume ratio of the sample, which is large in a vial. Methods of preventing evaporation include coating the vial with a membrane lid [29], saturating the headspace with water [37], working in an enclosed environment with increased humidity

[33], continuous compensation with solvent [34] and addition of glycerol [38]. However, the evaporation can also be a positive feature, for example when a large volume of a sample solution is available with a concentration giving poor detectability, it is advantageous concentrate the sample prior to analysis. This can conveniently be carried out in a vial through multiple additions of sample solution, allowing the solvent to evaporate between each addition [34].

3.1.3 Gel pad Arrays

Gel pad arrays can be used for the same purposes as DNA microarrays, but since the gel pad provides a three-dimensional porous and aqueous environment it also allows for novel applications, see figure 1 (c). Argonne National Laboratories has developed a MicroArray of Gel-Immobilized Compounds on chip (MAGIC chip) [39]. The MAGIC chips contain an array of gel pads that are separated from each other by a hydrophobic surface. Different compounds can be chemically immobilized in the gel pads. MAGIC chips containing immobilized oligonucleotides, DNA, and different proteins have been manufactured and tested for various applications [39].

The manufacture of the MAGIC chip consists of three steps: 1) immobilization of polyacrylamide gel pads (10x10x5 μm or larger) by photopolymerization on a glass slide, 2) selective addition of the probe solution, and 3) covalent immobilization of the probe within the gel pads. To avoid cross-contamination of different probes applied to adjacent gel pads, the pads are separated by a hydrophobic glass surface. To prevent the evaporation from the gel pads during chemical reactions, the chips can either be placed in 100% humidity, covered with a glass cover slip or an oil layer [40].

The gel pads provide more than 100 times greater capacity for immobilization than a two-dimensional glass support [40]. The high immobilization capacity increases the sensitivity of measurements [41], and allows for example kinetic and thermodynamic analysis by measuring the melting curves of duplexes [40]. Hybridization and other processes within the gel resemble a homogeneous liquid-phase reaction rather than a heterogeneous solid-phase reaction. These factors enhance the discrimination between perfect duplexes and mismatched ones. Hence, MAGIC chips have dual properties; they can be used as an array of immobilized compounds, or they can be used as an array of micro-test tubes holding about 0.2 nl or more to carry out different chemical and enzymatic reactions.

3.1.4 Bead Arrays

Recent reviews illustrate the rapid proliferation of DNA microarrays and describe the impact that the technology poses for the future [42-43]. Despite the success of the DNA microarrays the fabrication techniques remain a challenge for creating cost effective devices. While these microarrays have generated useful new genetic information and diagnostic capabilities, they suffer from both large feature and overall substrate sizes and require a new fabrication protocol to change any of the probe sequences in the array.

An alternative to DNA microarrays is an approach based on DNA probes bound to microspheres. The process of functionalizing beads with DNA probes is well established [44]. Different probe-functionalized beads can be analyzed simultaneously by labeling each bead type and mixing them. Identification of reactive beads in solution requires encoding each bead via chemical [44], spectrometric [45], electronic or physical means [46]. Although the production of such beads is less costly and elaborate than DNA microarray production, the analysis of beads in solution is more complex. In this section bead arrays will be presented where the simplicity of beads preparation is combined with the convenient DNA microarray analysis techniques.

The company Illumina Inc. (San Diego, CA) has taken an innovative approach to microarray design by combining fiber-optic bundles with specially prepared beads that self-assemble into an array, see figure 5. A typical fiber-optic bundle with a 1 mm diameter could contain 50 000 individual fibers. Dipping the bundle into a chemical solution etches microscopic wells at the end of each fiber. In a separate process, single-stranded oligonucleotide probes are immobilized on different optically encoded beads. The pure encoded, oligonucleotide functionalized bead populations are then mixed to form a stock solution. This library of beads is distributed randomly over the fiber array surface. The size of the beads (about 3 μm in diameter) matches the size of the wells so that only one bead occupies each well, see figure 5 (d). Upon drying, the beads are held firmly in the wells on the fiber-optic bundle. Since the bead array is randomly assembled, it must be decoded to determine which bead type occupies which well. When the bead array is ready the user dips it into the sample solution. This step is sufficient for the sample strands to find their matches on the coated beads. Sample binding can be quantitatively analyzed by shining a laser through the fiber-optic bundle and measuring the emitted light with a CCD attached to a microscope, see figure 5 (f). When the analysis is completed the beads can be removed from the fiber tip which can be regenerated.

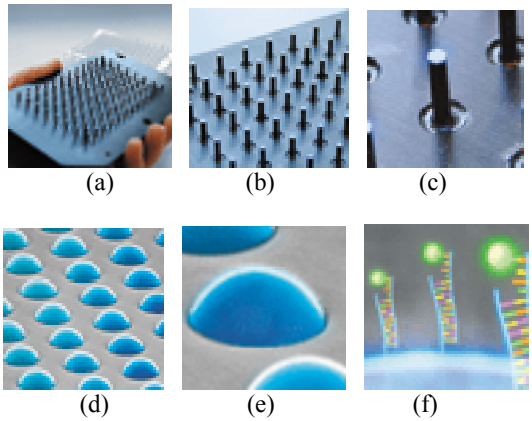


Figure 5. Illumina uses beads and fiber-optic bundles to assemble arrays of unprecedented density. The illustration depicts increasing levels of magnification (http://www.illumina.com).

By arranging the fiber-optic bundles to match the layout of the 96-, 384-, and 1536-well microplates an array of arrays, see figure 5 (a-b), capable of screening 3 million unique assays/microplate. Unlike most existing microarray technologies that allow researchers to screen one sample for thousands of genes, the bead arrays will address the growing demand for screening thousands of samples across only hundreds of genes, all on one bead array. The DNA bead arrays described above have smaller feature sizes and higher packing densities compared to DNA microarrays. The advantages of this high-density randomly distributed micrometer-sized bead-based DNA array include cost-effective production of the bead arrays in seconds, high-throughput analysis, easy replacement with different or additional beads when different testing is desired, and facile regeneration of the sensor and substrate. In addition, the array can be brought to the sample solution rather than the solution being brought to the array.

This thesis presents an alternative method for generating bead arrays (Paper 7). Instead of using an optical fiber with etched wells as a substrate, a dot array of self-assembled and self-sorted polymer beads for analytical and biochemical screening has been generated on unstructured silicon (Paper 7). Figure 6 shows a conceptual drawing of the different steps involved in generating the dot array. The dots are separated by a lithographically defined hydrophobic teflon-like film (C_4F_8) preventing cross-contamination between the dots (figure 6 (a)). The dots are then chemically modified by parallel microcontact printing (μCP), which is a novel technique developed in this study, enabling different chemical modification of different dots on the array simultaneously, see figure 6 (b). The stamp is

designed to modify each row of dots with the same compound. Beads with different functional groups are then self-arranged onto the matching dots, see figure 6 (c). Until today, fluidic self-assembly has been realized by using gravitational forces [47], minimization of interfacial free energy [48], capillary forces [49] or magnetic forces [50]. In paper 7, streptavidin-, amino- and hydroxy-functionalized microspheres are allowed to self-sort and self-arrange based on surface chemistry only, which has not previously been reported.

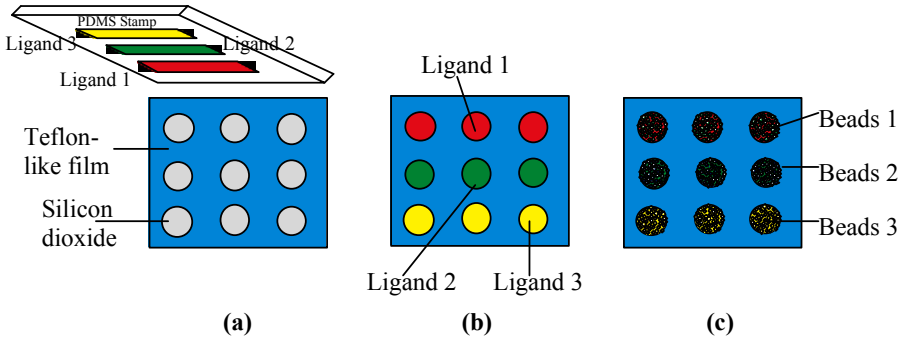
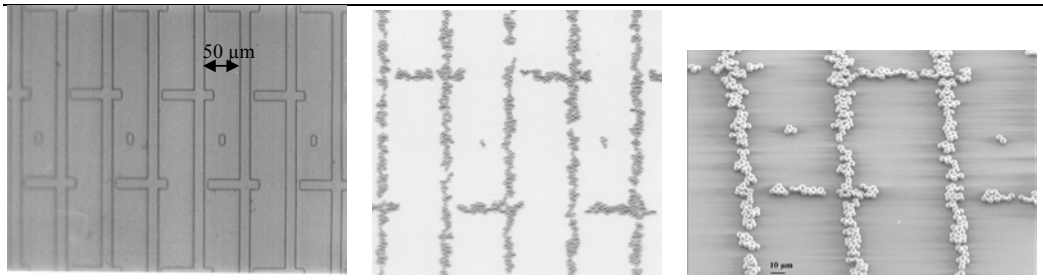


Figure 6. A conceptual drawing showing the different steps involved in generating the dot array.

The interaction between biotin and streptavidin is extensively used in analytical biochemistry [51], including DNA immobilization on solid support. In this study, biotin-labeled bovine serum albumin (BSA) is printed on the substrate surface and streptavidin is immobilized on the beads. Streptavidin-coated beads with a diameter of 2.8 µm have successfully been immobilized with a surface coverage of about 13±1 beads/100 μm^2. Beads have also been immobilized into a high resolution pattern, shown in figure 7 (a), by using a structured stamp with features as small as 5 µm. In figure 7 (b) it is shown that beads are successfully arranged into an almost perfect match of the printed pattern. The scanning electron microscope (SEM) image in figure 7 (c) demonstrates that features with as few as two-three beads can be formed reproducibly which corresponds well with the widths (5-10 µm) of the printed lines. The beads are firmly attached to the substrate and liquid can be added and withdrawn without problems. The immobilized beads can, however, be removed from the substrate by sonication in water for 30 seconds. Beads have also successfully been immobilized to quartz and polyethylene substrates using the same methodology.

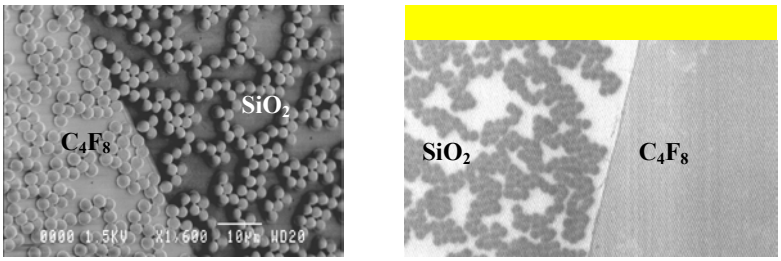


(a) **(b)** **(c)**

Figure 7. Streptavidin-coated beads (2.8 µm in diameter) immobilized in a high-resolution pattern on a biotin modified silicon surface. In (a) a photo of the stamp is shown. In (b) and (c) the beads are arranged into an almost perfect match of the printed pattern.

To investigate how well C_4F_8 functions as a passivating film, biotin-labeled BSA was printed on an interface of silicon dioxide and C_4F_8. The printed biotin-labeled BSA is barely visible in the SEM, the printing was therefore evaluated by immobilization of beads. Figure 8 (a) shows that the streptavidin coated beads absorb non-specifically on the C_4F_8. However, proteins are known to non-specifically attach to surfaces [52]. To remove the non-specifically adsorbed protein, the chip was sonicated in a 10% detergent solution for 5 minutes before immobilization of beads. In figure 8 (b) it can be seen that the protein was successfully removed since the beads are specifically localized to the printed area on the silicon dioxide only.

Hence, bead arrays on unstructured substrates can be formed in two ways, either a pattern is printed on the substrate and beads are allowed to self-assemble onto the printed pattern. Then there is no hydrophobic film separating the immobilized beads and the array would not be suited for analysis of different samples on the same chip due to cross-contamination, like in the DNA microarray case. The other possibility is to first generate a pattern by photolithography and deposit a passivating film, like C_4F_8. Then an unstructured stamp can be used to activate the silicon dioxide areas allowing beads to self-assemble onto the pattern generated by photolithography. The result is an array of immobilized single or multiple beads that are separated from each other by a hydrophobic film. Hence, the individual beads constitute reaction chambers where different samples can be analyzed with low risk of cross-contamination.



(a) **(b)**

Figure 8. (a) The printing of biotin-labeled BSA on C_4F_8 is evaluated by immobilizing streptavidin-coated beads showing that the BSA was non-specifically adsorbed onto the C_4F_8. (b) A photo showing that the printed biotin-labeled BSA was not non-specifically adsorbed onto the C_4F_8 since the beads are specifically localized to the printed area.

Depending on the kind of analysis to be performed on the bead array, biotin-labeled probes can be attached to the streptavidin-coated beads before or after [53] the beads are immobilized on the substrate. In the first case it would be convenient to use optically encoded beads as in the Illumina system. The latter case would involve robotics to load the beads with the different probes. Currently the bead arrays are being evaluated for their application in genomic analysis by performing DNA analysis using pyrosequencing [54].

Compared to DNA microarrays there are a number of advantages of the bead array described in this thesis, such as smaller feature sizes and higher packing densities, easy replacement with different or additional beads when different testing is desired, and that the surface area is increased when using a polymer as a linker. In addition, an appropriately selected polymer can allow a greater flexibility, *i.e.* rendering the system more gel-like (compared to the gel pad microarray). Together these properties imply more efficient reaction conditions and therefore facilitate miniaturization. Finally, since the beads are separated by a hydrophobic film different samples can be analyzed on the same chip. Compared to the Illumina system no etched wells are needed for assembly and the beads self-assemble purely based on surface chemistry.

3.1.5 Summary

The main advantage with using an open structure, such as a spot on an array or an uncovered vial, is the ease with which samples and reagents can be added or withdrawn. Other general advantages are the large number of spots/reactors which can easily be manufactured on a single chip and the lack of need for interconnections. The main disadvantage is the fast evaporation of small volume samples (nano or picoliters). Manipulation of small sample volumes can be facilitated by dilution to obtain a larger

volume, which is easier to handle. However, retaining a high concentration of reagents is important for fast reaction kinetics. Also, it is of great importance to maintain a high concentration of analyte to avoid detection difficulties when analyzing the sample. Other disadvantages are contamination from the outer environment (dust, including biological material) and sample contamination.

The different surface-based microarray systems described in section 3.1 all have their specific advantages and disadvantages. Which system is the most suitable will be decided by the specific applications.

3.2 Microfluidic Devices for Genomic Analysis

Microfluidic systems are devices where material is transported within miniature channels. There are a number of microfabricated devices in the literature ranging from single chips for polymerase chain reaction (PCR) to complete analysis systems. Ultimately they are more versatile than surface-based microarray systems, as microfluidic systems afford greater control of material location.

The advantages conferred by such microfluidic systems are numerous and wide ranging. Some of these are summarized in table 1 [12]. The reduction in size leads to less reagent consumption and ultimately the fabrication of such systems will be economically advantageous compared to traditional analysis systems. Other advantages arise as a result of the scaling of systems, giving dramatically increased performance. Improved thermal diffusion means that fast cooling and heating of fluidic elements may be accomplished. This also means that, for example, in electrophoretic separations, higher voltage gradients may be used without Joule heating of the system since the power is more efficiently dissipated within the microstructures. This gives improved speed and efficiency compared to conventional electrophoretic methods. The efficiency of electrophoretic separations, measured in the number of theoretical plates, is proportional to the length of the separation channel over the diameter of the channel. This means that reduction in size can be successfully facilitated without any loss in the number of theoretical plates.

Property	Advantage conferred	Reason
Reagent consumption	Reduced consumption and requirement	Reduced size requires less material.
Mass transfer (diffusion)	More rapid across the width of channels	The effective diffusion time of material across the width of the channels is proportional to d^{-2}, where d is the channel diameter.
Heat transfer	More rapid (improved cooling and heating times)	Effective rate of thermal dissipation is proportional to d^{-2}. Therefore, it increases as d decreases. This allows for higher driving voltages as heat is dissipated more efficiently.
Separation efficiency	Improved speed of separations	Higher voltage gradients may be employed, faster separations give less diffusional band broadening.
Flow	Generally laminar	Reynolds number is proportional to d. Therefore, it decreases as d decreases. Typical Reynolds number in microchannels are of the order of 1-200, well below 2000 (which is seen as the transitional point for turbulent flow).
Material transport	Often less external pumping and valving required	Electroosmotic control of fluid motion allows for valve-less systems. Capillary filling is also increased in hydrophilic microchannels.
Portability	Improved	Smaller systems, no external pumps required.
Parallelization	Readily achieved	Single chip can be designed with a number of separation channels or reactors.
Disposability	Improved	Single-use systems readily produced with low costs.

Table 1. Some advantages conferred with microfluidic systems adapted from [12].

Conventional DNA sequencing is based on the enzymatic chain termination method developed by Sanger [55]. This approach employs gel electrophoresis to separate the base-specific terminated DNA fragments according to their size. Pyrosequencing is a novel non-electrophoretic method that is based on coupled enzymatic reactions. In the following sections the miniaturization of capillary electrophoresis and pyrosequencing will be presented and compared.

3.2.1 Microfluidic Electrophoretic Devices

Microfabricated separation systems have become integral components for chemical analysis after the critical evaluation of the benefits of miniaturization was presented by Manz *et. al*. in 1990 [1]. DNA continues to be the main molecule of interest; other molecules being studied include RNA, proteins, and peptides. A noticeable trend in microseparations has been the dominance of electrophoretic techniques over chromatographic techniques. This trend is predominantly because pumping liquids in chromatographic separation systems requires systems that can withstand very high pressures while

electrophoretic systems can be operated by simply changing the applied voltages. Another significant trend is the constant push towards improving separation power (high resolution, faster analysis times) while keeping the separation lengths short.

Figure 9 shows a schematic of a basic chip-based device for electrophoretic separations. The channel defined by points 1 and 2 provides the separation channel and that defined by 3 and 4 is the injection channel. At the end of each channel, there are reservoirs for either waste, buffer or sample. These also provide access for the electrodes. The channels may be filled with sieving material such as polyacrylamide gel [56]. Applying a voltage between points 3 and 4 allows for sample material to be pulled across the cross-junction, switching off this voltage and applying one between 1 and 2 pulls material onto the separation channel. This system allows very small plugs (of picoliter volumes) of sample to be introduced; careful control of the driving voltages can produce plug widths smaller than the injection channel by pinching [57]. Plug sizes up to 10 times less than the equivalent for conventional capillary electrophoretic systems can be produced. Using an offset double T-injection system as shown in figure 9 (b), larger plugs (sizes defined by the geometry) can be produced.

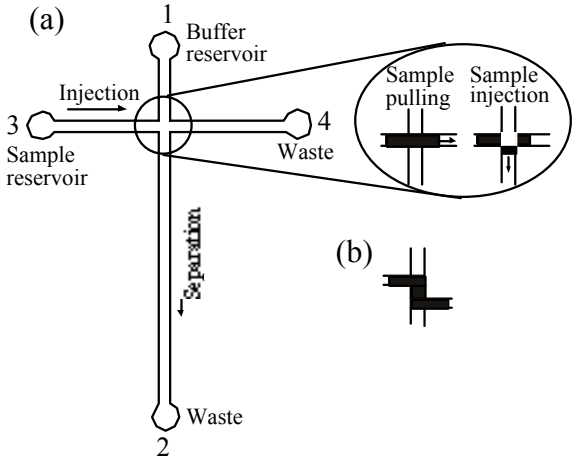


Figure 9. A schematic drawing of a typical chip-based electrophoretic system with (a) cross injection and (b) double T injection system. The sample plugs are depicted for an idealized case.

Equation 1 [58] gives an approximation of the theoretical resolution obtainable between two species in an electrophoretic system. In this equation, wall interactions and temperature effects have been ignored. It has also been assumed that adjacent peak widths are equivalent. The theoretical resolution R_T is given by,

$$R_T = (1/4)(\Delta\mu/\mu)L(\sigma^2_{inj} + 2DL/\mu E)^{-1/2} \qquad (1)$$

where $\Delta\mu$ is the difference in electrophoretic mobility between the species, μ the average mobility and σ^2_{inj} is the variance in the injections. *D* is the longitudinal diffusion coefficient of material being separated, *L* is the length to the detector from the injection point and *E* is the electric field. In polyacrylamide gel-based systems, it is important to note that both the diffusion coefficient and $\Delta\mu/\mu$ (a measure of the selectivity of the electrophoretic process), are a function of the gel type, temperature and field strength. $\Delta\mu/\mu$ is found to decrease with DNA fragment size for most gel systems, meaning that it is harder to separate longer fragments. More surprisingly, the diffusion coefficient of DNA in polyacrylamide gel is field dependent. A 400 base DNA chain in a field of 200 Vcm^{-1} has a diffusion coefficient that is roughly 2.5 times larger than that in no field. This effect is thought to be a result of the DNA changing shape in the field and is greater for longer DNA chains. For similar reasons, it is also found that $\Delta\mu/\mu$ decreases at high field strengths. These facts have important implications for DNA separations in microfabricated devices and, therefore designs must include a compromise between channel lengths and field strengths. It has been stated [58] that it is unlikely that it will be possible to sequence DNA fragments of over 400 bases in length in channels of less than 10 cm in length, since any gains found by increasing the field will be offset by losses of decreased selectivity and increased diffusional band broadening. Sizing of fragments, where resolution of 10-25% typically suffices (rather than the single base resolution required for sequencing), is possible in electrophoretic microfluidic systems for up to 10 000 bases.

Despite these disadvantages, the performance of microfluidic systems is far better than traditional slab gel or capillary devices. There is very little loss of performance due to Joule heating effects and the ability to inject very small well defined plugs means that the resolution of the system can be near the diffusion-controlled maximum (since σ^2_{inj} tends to zero as the plug size decreases).

The diffusion coefficient and $\Delta\mu/\mu$ of smaller DNA fragments are far less field dependent than longer DNA fragments. This means higher fields can be employed without brand broadening. Hence, for smaller fragments of DNA, much faster separations than those using traditional methods can be

routinely realized. Separations 100 times faster than slab gels and 10 times faster than capillary electrophoresis are readily obtainable. This has been shown for ultra-fast allelic profiling of short tandem repeats which are used in genotype testing [59].

Arrays of microfluidic electrophoretic devices are being developed [4, 60-62]. These potentially enable a further increase in the throughput by allowing parallel analyses. A system of 96 channels on a 10 cm diameter wafer has been reported [62]. A specially designed confocal scanner allows all the channels to be simultaneously monitored.

Detection capabilities can be enhanced by for example sample concentration. A system offering sample concentration before electrophoretic analysis has recently been reported [63]. The injection microchannel was adapted to incorporate a porous membrane just after the cross-junction. The porous membrane, made from silicate, is semi-permeable and allows ionic current to pass but not DNA. This can lead to an up to two orders of magnitude concentration of the DNA in the sample injection area.

Systems employing plastics rather than glass or silicon have also been developed. These should provide cheaper disposable systems. DNA fragments have been separated on poly(dimethylsiloxane) (PDMS) devices [64]. PDMS has a lower capacity than glass or silicon to dissipate Joule heating but this did not prove a problem with the device. Plastic injection molded microchips [65] made from acrylic material have been successfully employed to separate DNA with a 2-3% variability.

Microseparation systems currently rely on one of the three major detection modes: fluorescence, electrochemical or chemiluminescence detection [66]. Because of its sensitivity, laser induced fluorescence (LIF) is by far the most popular detection technique for microchip-based electrophoresis. However, the linear dependence of optical detection techniques on the detection path length, as well as the size and complex nature of LIF detection, conflict strongly with miniaturization. For practical purposes, an even more important drawback of LIF is that most biologically relevant compounds require labeling with a fluorophore prior to being amenable to LIF detection. This complicates the overall analytical procedure as well as chip design. Electrochemical detection, especially conductivity detection, provides an alternative detection method and allows detection of many compounds without derivatization at comparable sensitivity to LIF. Electrochemical detection is ideally suited for the microchip format since microfabrication of the detection electrodes generally results in higher sensitivity and faster response times [67-68]. Capillary electrophoresis microchips with integrated electrochemical detectors have been presented [69-70].

A number of companies are beginning to commercialize microfluidic DNA chips for electrophoretic separations. Caliper Technologies Corporation and Agilent Technologies have released an instrument

that uses disposable glass chips for integrated analysis of nucleic acids, providing sizing and concentration information within 90 seconds, see figure 10. Aclara Biosciences Inc. intends to provide microfluidic systems where ready-to-use plastic chips are pre-filled with gel that can be used for rapid DNA fragment sizing, see figure 11.

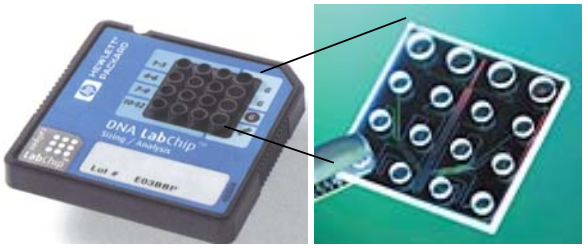


Figure 10. The LabChip from Caliper Technologies (http://www.calipertech.com).

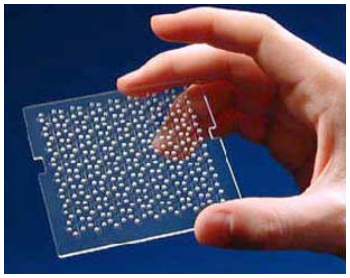


Figure 11. The plastic LabCard from Aclara Biosciences (http://www.aclara.com).

As the separations on microchips become more challenging, higher absolute separation efficiencies are needed. This is most easily accomplished by increasing the separation channel field strength. The field strengths on microchips, however, have reached the point where Joule heating has become a concern. An alternate method to improve the absolute separation efficiency is to increase the separation channel length while retaining the high field strength in the separation channel. However, to maintain the compact footprint of the microchip, the lengthened separation channel must incorporate turns which will add geometrical contribution to the analyte dispersion. This excess dispersion occurs both as the migration distance and field strength vary laterally across the width of the channel in the turns. To minimize turn-induced band broadening, turn designs in which the turn is tapered before the turn and widened after the turn have been examined by Paegel *et. al*. [71].

The most striking common feature of microchip-based electrophoresis systems are the fast analysis times. The fact that no external pumps and valves are required, since fluid flow can effectively be controlled by simple switching of electrical potentials, makes this technique particularly suitable for automation and miniaturization. However, if the electrodes are inserted into the fluidic circuit generation of bubbles at the electrodes from electrolysis gases can disconnect the electrode from the buffer solution [72]. Caliper Technologies Corporation uses a combination of pressure/vacuum and electrokinetic forces to move fluids and do separations on chips. The combination of electrokinetic and pressure actuation provides a powerful means of fluid movement because it transcends the limitations of either method alone.

3.2.2 Microfluidic Devices for Pyrosequencing

Pyrosequencing is a sequencing-by-synthesis method developed by [54, 73] which relies on sequential addition and incorporation of nucleotides in a primer-directed polymerase extension. The principle of pyrosequencing is outlined in figure 12. The DNA fragment of interest (hybridized to a sequencing primer) is immobilized to a solid-phase (*i.e.* beads) and mixed with DNA polymerase, ATP sulfurylase, firefly luciferase, and a nucleotide-degrading enzyme such as apyrase. The four nucleotide bases are added to the mixture in a defined order, *e.g.* CGAT. If the added nucleotide forms a base pair to the primer-template, the DNA polymerase incorporates the nucleotide and pyrophosphate will consequently be released. The released pyrophosphate will then be converted to ATP by ATP sulfurylase and luciferase will use the generated ATP to generate detectable light. The excess of the added nucleotide will be degraded by apyrase. However, if the added nucleotide does not form a base pair to the DNA template, the polymerase will not incorporate it and no light will be produced. The nucleotide will rapidly be degraded by apyrase and the next nucleotide is added to the pyrosequencing reaction mixture.

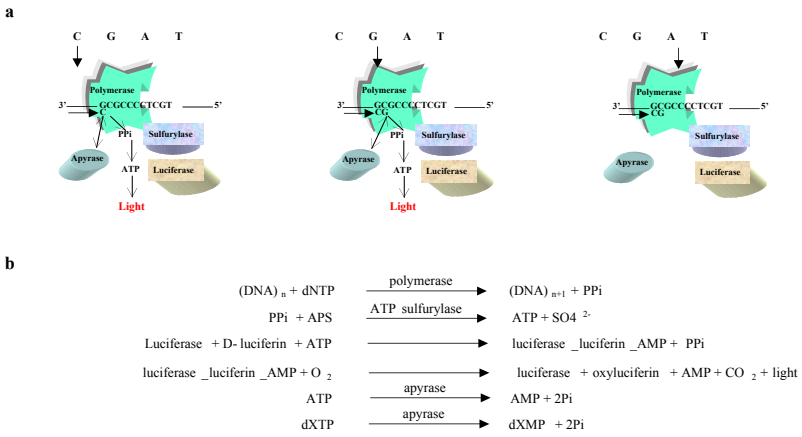


Figure 12. The principle of pyrosequencing from [74].

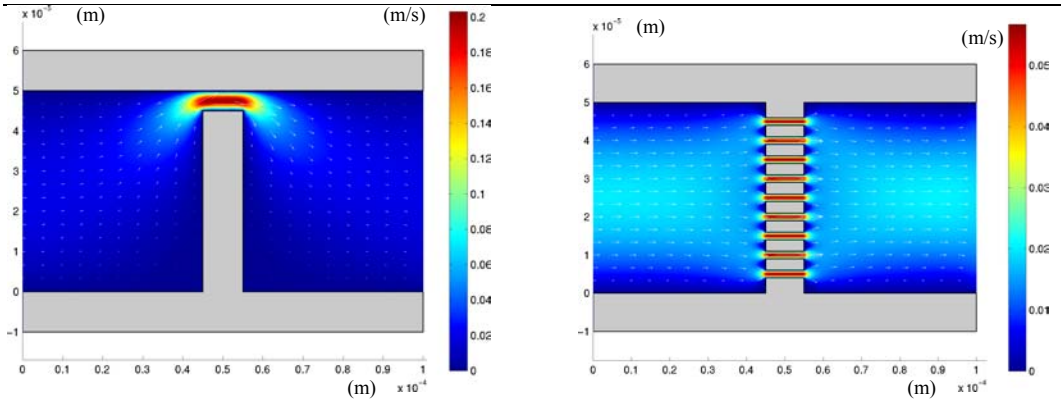
This novel approach has several advantages compared to standard sequencing methods such as real-time detection, no labeling is required, it is suitable for handling multiple samples in parallel, and no electrophoretic separations are needed.

At present, microtiter-based pyrosequencing allows sequence determination of 30-40 bases. However, there are some inherent limitations with this technique which arise when more than 30-40 bases are to be sequenced. Product accumulation, enzyme impurities and decreased enzyme activities could explain these limitations. Another drawback of the pyrosequencing method is that the reagents are expensive, hence the cost per sequenced base is not competitive with other techniques such as capillary electrophoresis.

To address some of the above mentioned problems a microfluidic filter-chamber device for performing pyrosequencing on beads in microscale has been developed (Papers 1, 3, 9).

Micromachined Filter-Chamber Device

The combination of beads and microfluidics is a powerful analytical tool. By using beads as the solid phase the reactive surface area in a flow chamber can be increased and easily chemically modified. To perform and detect chemical reactions on beads in microfluidic devices, the beads must be trapped into a constrained volume. Several designs of mechanical barriers in different materials have been presented. The most common design consists of a dam of either glass [75], quartz [76], or plastics [77]. However, in these devices there are parts of the trapped bead volume where the flow velocity is low. Therefore, this design is not optimal for performing chemical reactions on the captured beads. This assumption is based on flow simulations of a dam design without beads, see figure 13 (a). By using filter pillars as mechanical barriers in a silicon channel [78] the flow-through at the bead trapping location is more homogeneous, which can be seen in the flow simulations in figure 13 (b). Hence, this design provides better chemical reaction conditions since a larger fraction of the trapped beads will be exposed to the samples and reagents that are flushed through. However, the number of pillars is limited by the channel width, which means that for a narrow channel the risk for clogging is high.



(a) **(b)**

Figure 13. Flow simulations showing the flow velocities in (a) a dam filter and (b) in a pillar filter design. The initial flow velocity was set to 2 µl/min. The slot opening in the dam design is 5 µm and in the pillar design the pillars are 3 µm wide with a spacing of 2 µm. The channel depth is 50 µm in both cases. The flow simulations were performed using the FEMLAB software package from Comsol (Courtesy: Per-Olof Persson, Comsol, Stockholm, SE).

An alternative method for capturing beads without the use of mechanical barriers is to use external magnets [79] or on-chip integrated magnets [80]. Since mechanical barriers are not needed to trap the beads the clogging risk is also reduced. However, magnetic beads must then be used which sometimes is a limitation due to the iron content and opaque color. In addition, external magnetic systems complicate precision handling and result in a bulky system. Incorporation of magnetic components on wafer level results in a complicated fabrication process.

To enable chemical reactions on non-magnetic and magnetic beads this thesis presents a flow-through filter-chamber for bead trapping (Paper 1). In this design pillars define a square reaction chamber in which the beads are trapped. A larger number of pillars constitute the filter-chamber compared to a filter in a channel, which results in a less clogging sensitive filter. A schematic of the complete filter-chamber device is shown in figure 14. The beads are applied at the inlet and collected in the reaction chamber. The waste chamber surrounds the reaction chamber and is connected to the outlet. The device design is uncomplicated and is batch fabricated by deep reactive ion etching (DRIE). It is sealed by anodically bonded Pyrex to enable real time optical detection.

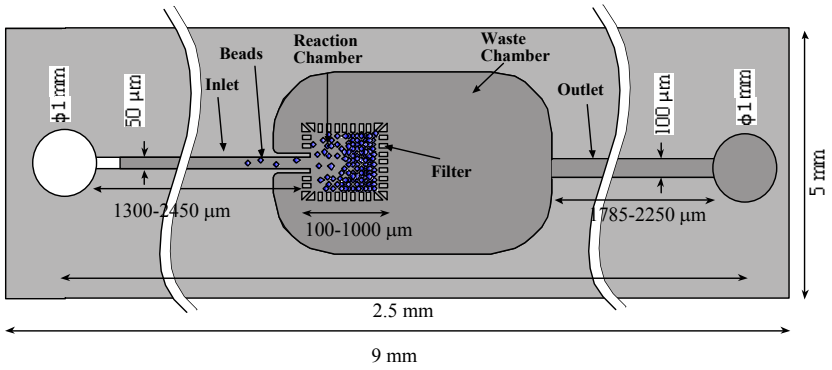
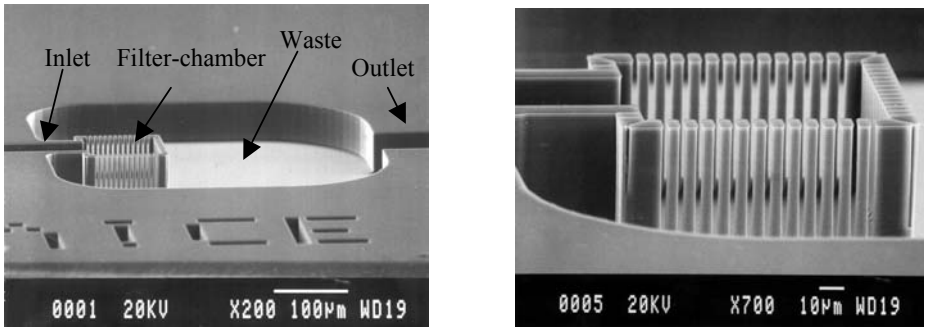


Figure 14. A schematic of the filter-chamber device.

Figure 15 (a) shows a SEM photo of the filter-chamber device defining inlet channel, filter-chamber, filter pillars, waste chamber and outlet channel. The side view of the filter-chamber at a higher magnification is shown in (b). The smallest filter-chamber design has a volume of 0.5 nl and can hold about 5740 beads with a diameter of 5.50 µm.



(a) **(b)**

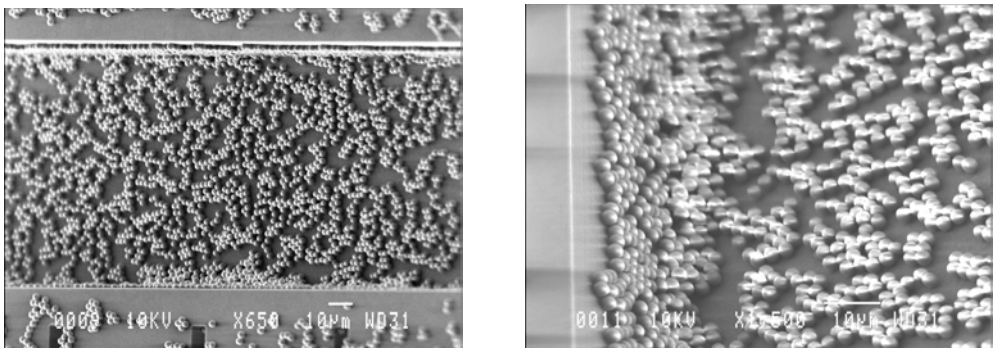
Figure 15. (a) A SEM overview of the filter-chamber device. (b) A side view of the filter-chamber. The pillars are 3 µm wide and 50 µm high with a spacing of 2 µm.

To investigate the fluid behaviour of the filter-chamber device, water without beads was first applied. The flow rate for water was about 3.5 µl/min when a constant pressure of 3.0 kPa was applied at the inlet. The flow rate for water was about 2.2 µl/min (when a constant pressure of 3.0 kPa was applied at the inlet) when the filter-chamber is completely packed with beads. Hence, the flow rate decreases by approximately 40% when the filter-chamber is packed with beads. When performing chemical reactions on the beads in the filter-chamber it is important that the flow resistance remains low

when the filter-chamber is packed with beads. Otherwise, it would be difficult to pump the reactants through the reaction chamber. To control the flow in the filter-chamber a manual syringe or an internal/external pump is needed.

Clogging of the filter-chamber is rare and can easily be remedied by applying back-pressure. Gas bubbles present in the samples did not affect the device performance. The beads can easily be removed from the reaction chamber by applying back-pressure. After removing the beads and carefully cleaning the micromachined flow-through device, it can be reused.

To selectively trap nonmagnetic beads in a monolayer in microfluidic devices without the use of physical barriers, a new technique is presented in paper 8. It is a fast, convenient and simple method, involving microcontact printing and self-assembly, that can be applied to silicon, quartz or plastic substrates. In the first step, channels are etched in the substrate. The surface chemistry of the internal walls of the channels is then modified by microcontact printing (Paper 6). The chip is submerged in a bead solution and beads self-assemble based on surface chemistry and immobilize on the internal walls of the channels, see figure 16. Finally, the channels are sealed with a PDMS film, which is applied like an adhesive tape. The device can then directly be used for screening or the immobilized beads can be further functionalized for analysis of DNA [53], for example.



(a) **(b)**

Figure 16. (a) SEM photo showing a 100 µm wide and 50 µm deep channel with immobilized streptavidin-coated magnetic beads (2.8 µm in diameter). The surface coverage on the bottom of the channels is about 14 beads/100 µm^2. (b) The surface coverage of beads on the channel wall is good except for the upper part of the channel where no beads are immobilized.

SNP Analysis Using Pyrosequencing

One of the application fields for pyrosequencing is analysis of single nucleotide polymorphisms (SNPs). It is believed that SNPs are associated with genetic diseases [81]. As the number of identified SNPs increases, there will be an increasing demand for efficient methods to type these sequence variations. Therefore, the possibility of using the filter-chamber device for SNP analysis using allele-specific extension by the pyrosequencing chemistry has been studied (Paper 9).

The principle of allele-specific extension for SNP analysis is shown in figure 17. PCR is performed on a polymorphic position and single-stranded DNA is obtained after immobilization on beads. The single-stranded DNA is divided into two tubes and addition of 3'-end allele-specific primers allows hybridization. The primers are designed to match one allele perfectly but mismatch the other allele at the 3'-end. In this way, each allele-specific primer-template hybridization provides information on the presence or absence of one allele.

Figure 18 is a schematic of allele-specific extension by the pyrosequencing reaction. If the 3'-end of the primer matches to the DNA template (a) and a pyrosequencing mixture containing all four nucleotides is added to the annealed template, a light signal will be produced. This signal indicates that the DNA polymerase has used the nucleotides to extend the primer and that the released PPi has been converted to ATP and then to light. If the 3'-end of the primer does not match to the DNA template (b), the DNA polymerase can no extend the primer and no light will be produced.

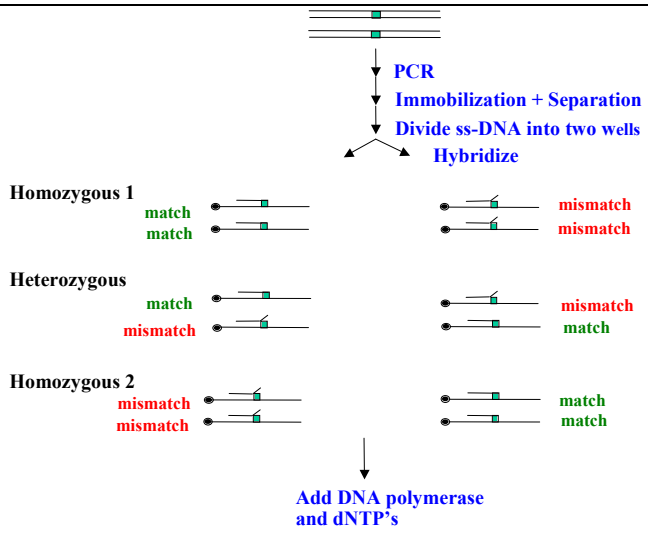


Figure 17. The principle of allele-specific extension for analysis of SNPs. Single-stranded DNA template immobilized on beads is prepared by treating the amplified PCR product. The template is divided in two wells and hybridization with the primers is performed.

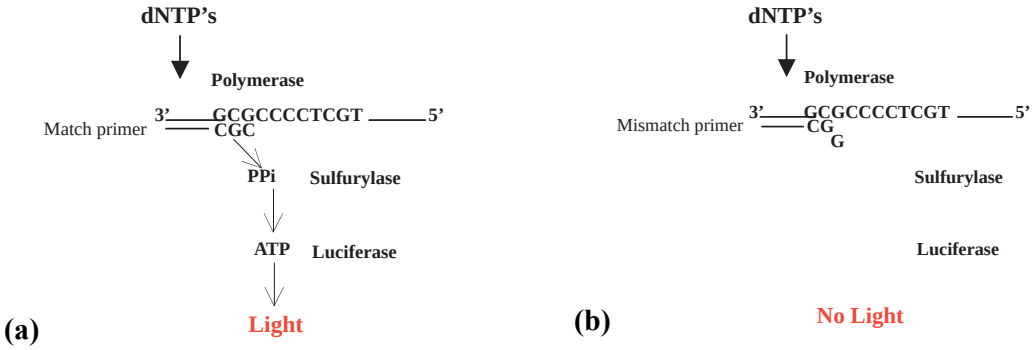


Figure 18. The principle of allele-specific extension using the pyrosequencing reaction. (a) 3'-end match primer annealed to the single-stranded DNA will result in detectable light. (b) 3'-end mismatch primer will not be extended by DNA polymerase resulting in no light.

This principle was used to analyze two SNPs located on chromosome 17p (codon 72 of the p53 gene) and chromosome 9q (wiaf 1764) (Paper 9). In figure 19 the results for the three possible variants of the SNP at codon 72 are shown. The coding SNP at codon 72 involves either a G or a C residue. The match and mismatch signals correspond to the matched and mismatched primer-templates in figure 18. In the case of homozygous G (figure 19 (a)) the match variant is extended and produces light that is successively increasing and can be distinguished from the mismatch variant. The light is detected by a CCD camera and is increasing with time because more PPi is released during the expansion of the primer. The mismatch variant is almost constant at low levels, revealing that the DNA polymerase is not able to extend the mismatched primer-template. In the case of heterozygous (figure 19 (b)), both alleles are extended which is expected since both alleles are present in a match format for both primer variants. It is notable that both extension signals follow the same pattern. In the homozygous C situation (figure 19 (c)) the match variant is extended and produces light while the mismatch is discriminated. The difference in light intensity between the match and mismatch variants is not as distinguishable as for homozygous G case. However, it is possible to distinguish the match and mismatch samples.

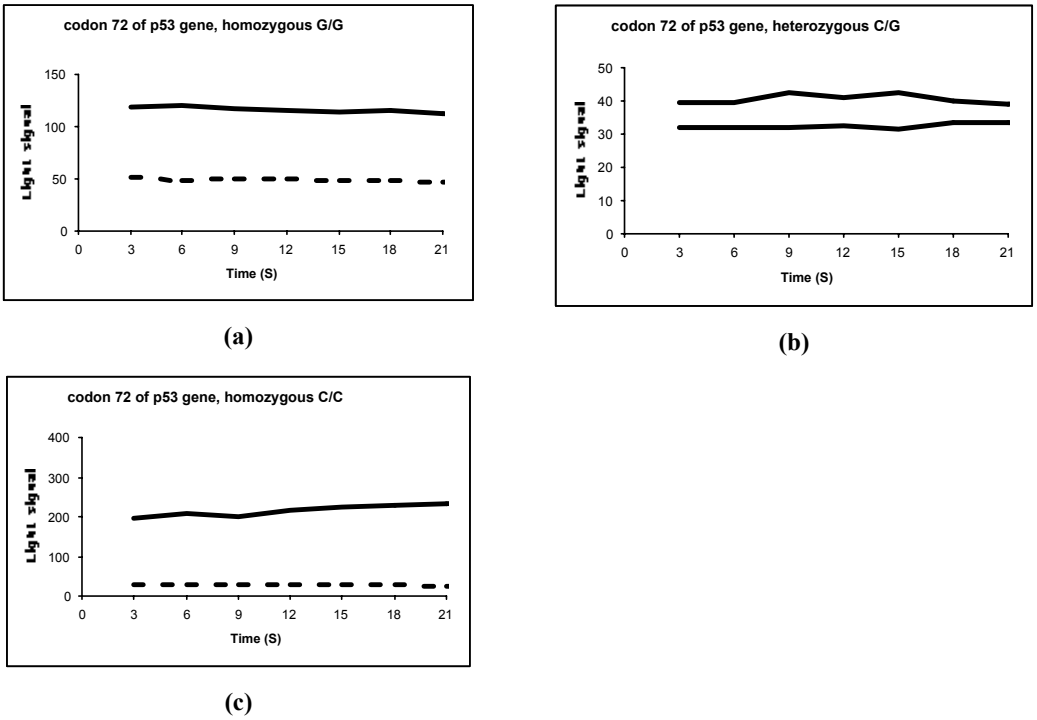


Figure 19. The results of allele-specific extensions in the filter-chamber device for codon 72 of the p53 gene.

Figure 20 shows the results for the three possible variants of the SNP at chromosome 9q (wiaf 1764), the possible match and mismatch configurations involve either a G or a T residue. In the case of homozygous G (figure 20 (a)), the match variant is extended and can be distinguished from the mismatch variant. For the heterozygous variant (figure 20 (b)) both alleles are being extended producing light at almost the same level. In the case of the homozygous T (figure 20 (c)) the match and mismatch samples are also distinguishable. However, the difference of the light intensity between the match and mismatch variant is smaller compared to the homozygous G case.

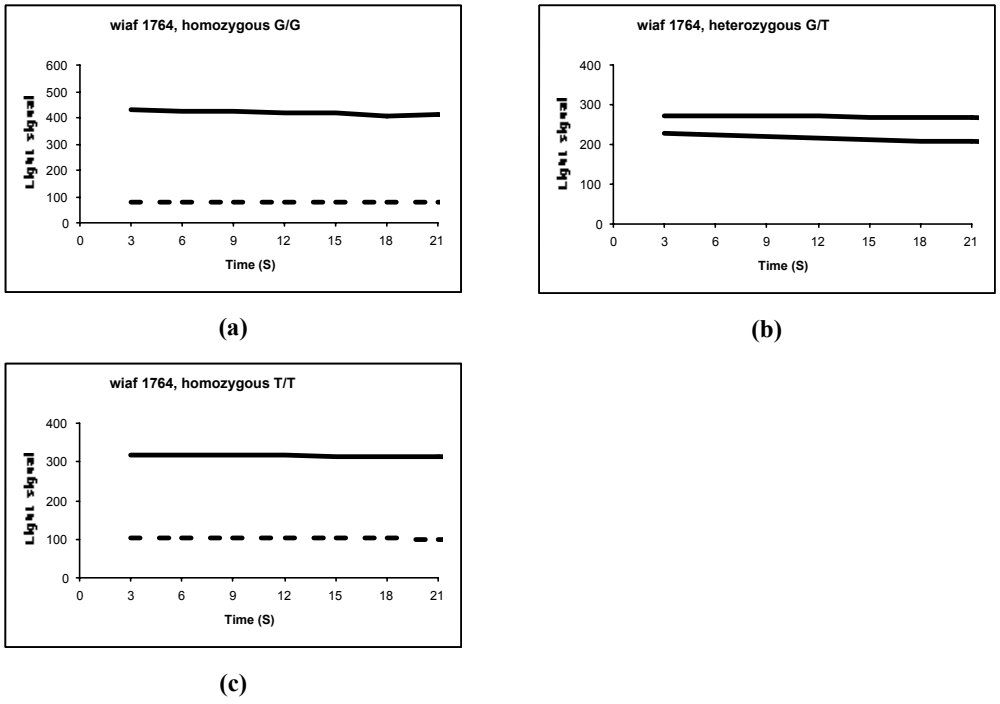


Figure 20. The results of allele-specific extensions in the filter-chamber device for wiaf 1764.

The above presented results clearly demonstrate that all the different SNP variations at the two sites could reproducibly be scored in the flow-through filter-chamber within a volume as small as 12.5 nl. This leads to 4000-fold reduction of the reaction volume compared to the present standard volume. Hence, the cost per assay can be dramatically reduced by using the microfluidic filter-chamber device. Since the filter-chamber is a flow-through device it is most probable that by-product accumulation is reduced which may lead to better performance, *i.e.* longer read lengths. However, since there are no

valves in the design precise control of the added reagents were difficult to achieve. Hence, the starting point of the chemical reactions taking place when the reagents reach the beads in the reaction chamber was unfortunately not exact, since some of the reagents might diffuse in to the reaction chamber before the suction at the outlet was applied. Therefore, a liquid control element at the inlet is required to perform more complex analysis in the filter-chamber device, (*i.e.* single nucleotide analysis using pyrosequencing).

Hydrophobic Valves

Some of the main requirements of the liquid control element to be integrated with the filter-chamber device include: it must allow multiple use and long term stability, be clogging insensitive (since beads are used), have a simple fabrication process which is compatible with the filter-chamber fabrication process, and it must be able to handle the wide variety of liquids used in biochemistry. Passive fluid control was achieved by modifying the surface properties of silicon dioxide, glass and plastics and has several advantages over many active fluid control methods such as pumps and valves. For example, no moving parts are needed resulting in a less complex and clogging insensitive system. However, the passive fluid control elements presented so far have limitations in either the design, temperature or chemical resistance [82-83].

Therefore, a new passive fluid control method that is based on lithographically defined hydrophobic patches in DRIE silicon channels using octafluorocyclobutane (C_4F_8) that fulfills all the above requirements was developed (Paper 2). Some of the advantages of this process are that no specific cleaning of the substrate is required, C_4F_8 is deposited both on the sidewalls and the bottom of the channels, a standard photoresist mask can be used to define the patches and that the fabrication process is fast, convenient, and dry performed using the passivation step in a standard ion coupled plasma etcher. To seal the device a low temperature adhesive bonding technique for fluidic devices was developed (Paper 5).

An inlet pressure of 760 Pa was needed for water to overrun the hydrophobic patch. For any application purpose, the 760 Pa valve pressure can easily be applied by an internal or external pump, or using a syringe. The suitability of using C_4F_8 as hydrophobic material for microfluidic biochemical applications was also shown (Paper 2). The C_4F_8 hydrophobic patches were therefore chosen to be included in the filter-chamber device as the passive fluid control element.

Filter-Chamber Array with Hydrophobic Valves

To enable parallel single nucleotide analysis of multiple DNA samples a filter-chamber array with hydrophobic valves at the inlets was developed (Paper 3). The design, shown in figure 21, consists of three filter-chambers and three hydrophobic valves, thus including several additional features compared to the separate filter-chambers and hydrophobic valves. It enables parallel sample handling with a simplified interface and time controlled analysis.

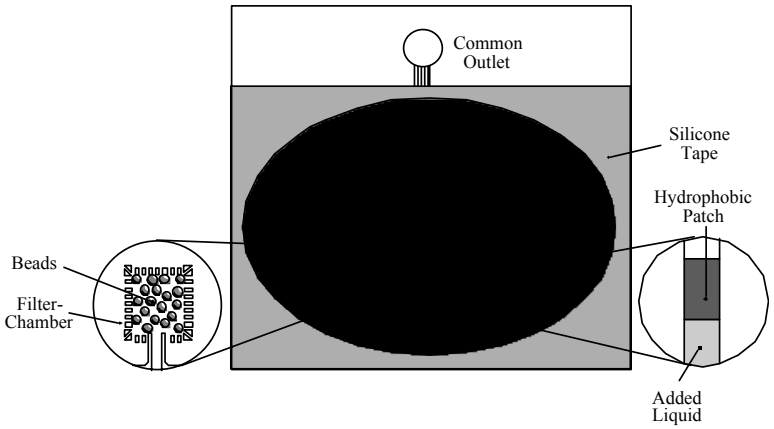


Figure 21. A conceptual drawing of the filter-chamber array. The two key elements (the filter-chamber and the hydrophobic valve) are magnified.

The filter-chamber array could be operated as follow: in the first step a liquid drop containing beads, sample 1, is applied at the inlet, see figure 22 (a). This liquid will (capillary) fill the inlet channel until the liquid reaches the hydrophobic patch (b). If suction (generated by a syringe) is applied at the outlet, increasing the pressure drop over the liquid-air interface, the liquid will break through the hydrophobic barrier and fill the filter-chamber (c). If the suction is maintained over a longer period of time, all liquid present at the inlet can be transported through the filter device, leaving the beads trapped. When the suction is removed (d), no liquid can flow back towards the inlet because the hydrophobic patch hinders such movement (e).

In the second step, a new liquid (sample 2) can be applied at the same inlet (f). The liquid is again stopped from entering the device by the hydrophobic patch. The air pocket at the hydrophobic patch functions as a physical barrier between sample 1 and 2. When suction is applied (g), sample 2 will cross the barrier and enter the filter-chamber where it can react with the beads. Hence, the second suction step functions as a time trigger for the analysis. This means that after loading sample 2, the user of the system has time to load the chip on the detector and prepare the measurement.

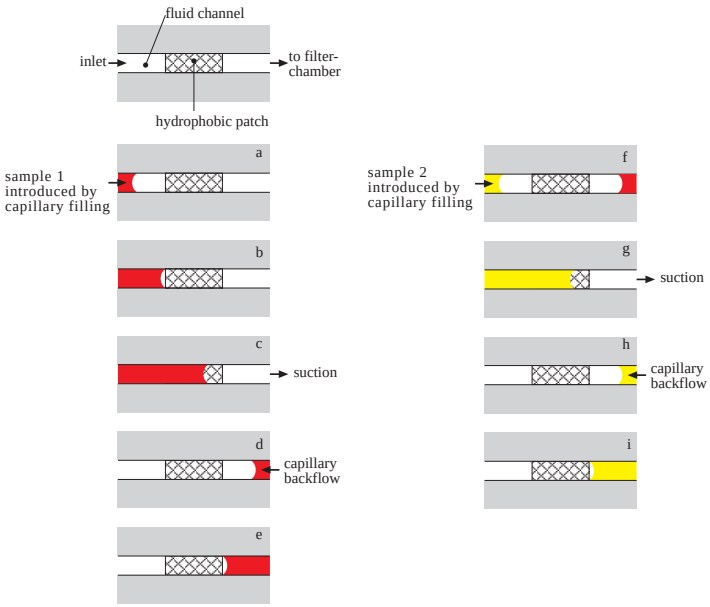


Figure 22. Illustration of the flow control achieved by using hydrophobic valves and external suction.

Both filter-chamber arrays with and without hydrophobic valves were fabricated and evaluated (Paper 3). Arrays without passive valves were first studied. The three filter-chambers, each holding 3.0 nl, were simultaneously filled with beads without complications. However, when the suction at the outlet was stopped some beads tended to flow back towards the inlets. Hence, in this design, as well as in the single filter-chamber device, it is not possible to precisely control the trapped beads or the addition of different reagents because of capillary filling and diffusion.

In figure 23, a photo of a filter-chamber together with a hydrophobic valve constituting a part of an array is shown. Since the passive valve can resist beads in solution the problem observed in the other array design, where the beads flow towards the inlet after the suction at the outlet is stopped, is solved in this design. Time controlled sequential addition of different reagents to the trapped beads was successfully realized in all the filter-chambers in parallel. Hence, more complex chemical reactions can be performed in the arrays with passive valves than in the arrays without valves. The results indicate that the three-chamber structure can be extended to a full array system including many more filter-chamber units (about 100 filter-chambers/cm^2).

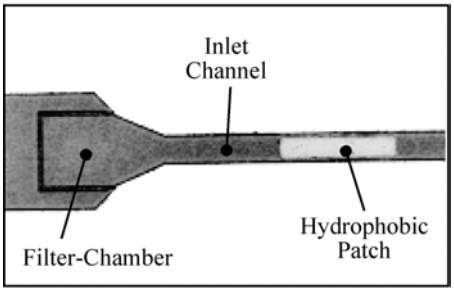


Figure 23. A photo of a part of the array showing one filter-chamber and hydrophobic valve. The hydrophobic patch is 200 µm long and the filter-chamber holds 3.0 nl.

In summary, the main advantage of the filter-chamber device for pyrosequencing compared with microchip-based electrophoretic systems for DNA analysis is that since no separation is needed for analysis the chip designs can be made more compact. In addition, no labeling is required which facilitates the sample preparation. This is important for the future design of an integrated system with sample preparation and analysis on the same chip. Today, off-chip sample preparation is the most time-consuming and labor intensive procedure in DNA analysis.

The fact that no excitation source is needed will also play an important role in the development of a miniaturized integrated system for pyrosequencing. Even though the filter-chamber device is reusable there is a demand for disposable devices from industry. Therefore, a technique for fabricating the filter-chamber device in plastics is underway. Currently, the filter-chamber array is being evaluated for performing single nucleotide analysis using pyrosequencing. The goal is to achieve longer read lengths than is possible in the microtiter-based systems used today.

SNP analysis using pyrosequencing on the immobilized monolayers of streptavidin-coated beads (Paper 7 and 8) has been initiated and the results will be compared with the ones achieved in a filter-chamber to determine where the detection limit is, *i.e.* if a monolayer of beads is sufficient to perform the analysis.

Another approach for miniaturization of pyrosequencing has been presented by the company Gyros AB (Uppsala, Sweden). Their technology enables the use of high throughput disposable compact discs that employ centripetal driven microfluidics with stop-flow functionality [77]. The flow in the filter-chamber device must be controlled by pressure generated by an internal or external pump. Therefore, the next section in this thesis will present a valve-less micropump which is suitable for integration with the filter-chamber device (Paper 4).

Micropumps

Micropumps are essential components in microfluidic analysis systems. A miniaturized laboratory (lab-on-a-chip) must be able to handle fluids with a wide variety of properties, *i.e.* viscosity, density, ion strength, pH, temperature, and surfactants. The micropump's sensitivity to fluid properties is dependent on the principle used for fluid movement. Today, many presented micro chemical analysis systems are based on electroosmotic and electrohydrodynamic pumping. The underlying pump principles build on the specific fluid properties of the pumped medium [84-85]. Electrohydrodynamic pumps use the kinetic energy of ions present in the liquid to create the pump action and electroosmotic pumps use the presence of immobilized surface charges, mobile charges in the sample and an externally applied electrical field. Thus, these pumps are inherently dependent on the properties (pH, ionic strength) of the pumped medium, making them unsuited for a large number of biochemical and biological liquids [5]. Therefore, the suitability of a new valve-less micropump in biochemistry has been shown (Paper 4).

The valve-less diffuser pump consists of two diffuser elements connected to a pump chamber with an oscillating diaphragm. The key components of the micropump are the flow directing diffuser elements. One diffuser element is directed from the inlet to the pump chamber and the other diffuser element from the pump chamber to the outlet as illustrated in figure 24. The oscillating diaphragm forces the fluid through the two diffuser elements. The result is a net transport of fluid from the inlet to the outlet due to the difference in the flow resistances in the forward (diffuser) and reverse (nozzle) directions (figure 24). Some of the main advantages of the diffuser pump are the absence of moving parts (excluding the pump diaphragm), the uncomplicated planar design, high pump performance, in terms of pressure head and flow rate, and the ability to pump a wide variety of fluids.

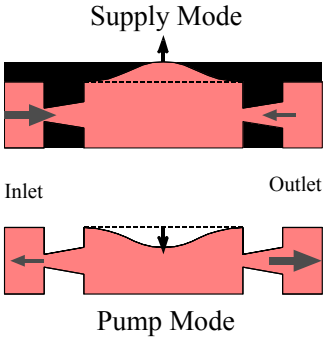


Figure 24. Working principle of the valve-less diffuser micropump.

Fluids encountered in various biochemical methods, see table 2, that are problematic for other micropumps, have been pumped with good performance. Bi-directional pumping was achieved for all the liquids by altering the frequency and amplitude. Hence, the flow directing capability of the diffusers are frequency and amplitude dependent. Fluids with higher viscosity have lower pump rates. However, no clear correlation between the pump rate and the density of the pumped fluids could be found. The pump performance of the valve-less micropump does not depend on the pH of the pumped media at all. Electroosmotic pumps, for example, are dependent on the ionic strength of the pumped medium. If a network of intersecting channels is used to pump samples of different ionic strength, the electroosmotic flow will become unbalanced and cause problems in the system [86].

Property	Range	Application Source
Viscosity	$1x10^{-3}$–$11x10^{-3}$ N s/m^2	50% glycerol ($11x10^{-3}$ N s/m^2) for handling of enzyme stock solutions
Density	1-2 g/mL	50% glycerol (1.25 g/mL) for handling of enzyme stock solutions
Ion Strength	0-1 M	monovalent salt for hybridizations
pH	1-6 6-13	Acetic acid for neutralization NaOH for denaturation
Temperature	-20 to 94 °C	Long term storage and DNA denature
Surfactants	0.05-1.0%	Tween for minimization of surface adhesion

Table 2. Fluid properties encountered in molecular biology.

The flow pattern inside the pump chamber was visualized by low concentrations of micro beads in the liquids. Vortex-like flow patterns occur in the pump under all conditions. The number of vortices and the direction in which they turn is dependent on driving frequency and diaphragm amplitude. Generally, the higher frequencies and/or diaphragm amplitudes result in a higher number of vortices and higher flow velocities. The observed flow patterns in the pump chamber indicates that the micropump could function as a micromixer if two inlets are fabricated [87].

The flow patterns described above have a strong influence on the gas bubble behaviour inside the pump chamber. Bubbles present in the pumped medium or formed in the pump chamber are washed out of the pump chamber by the vortices. Hence, bubbles do not affect the pump action significantly.

High concentrations of micro beads of two different sizes, 2.7 and 5.5 μm in diameter, have been pumped. The results show that the pump performance is insensitive to particles (∅<5.50 μm) in the pumped media.

In the great majority of mircofluidic devices, especially those that use electrophoretic separations, fluid flows are driven and directed using electroosmotic flow. Electroosmotic pumps offer a number of advantages over pressure driven pumps such as ease of fabrication and the absence of moving parts. Moreover, sample plugs suffer little from dispersion. However, electroosmotic pumps also have several disadvantages such as being inherently dependent on the properties of the pumped medium and very sensitive to contamination in the pumped medium. Since the filter-chamber device for pyrosequencing does not involve any electrophoretic separations the best choice is probably to use an external pump or to integrate the valve-less micropump at the outlet.

A novel tool in microfluidics is presented in paper 10. Expandable microspheres (Expancel, SE) are small spherical plastic particles, which consist of a polymer shell encapsulating a hydrocarbon liquid. When the microspheres are heated the liquid transforms into a gas, which increases its pressure. Together with softening of the thermoplastic shell this results in a dramatic irreversible increase in the volume of the microspheres, see figure 25. When fully expanded, the volume of the microspheres increases more than 60 times. Expandable microspheres are today used as blowing agents in many areas such as printing inks, paper, textiles, polyurethanes, and PVC-plastics [88].

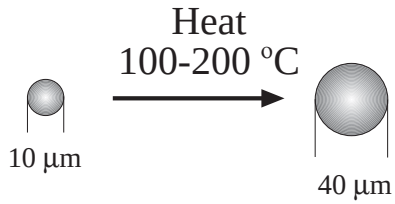


Figure 25. A schematic of the expansion principle of the expandable microspheres.

A prerequisite for using expandable microspheres as a tool in microfluidics is the ability to selectively place them in a predefined area of a device. In paper 10, two methods for selective immobilization of expandable microspheres have been studied for their application in microelectro mechanical systems (MEMS), including patterning by photolithography and microcontact printing combined with self-assembly based on surface-chemistry. After the immobilization step the microspheres were expanded. The actuation principle for the expansion can be classified as thermal. A heater transfers thermal energy to the microspheres, which subsequently expand. The expansion can be used to displace a liquid volume, generate a pressure or enlarge the internal reactive surface of a microfluidic system. The actuation is irreversible, *i.e.* the expandable microspheres maintain their expanded volume/pressure when cooled down again.

Figure 26 (a) shows selectively deposited expandable microspheres (before expansion) on a silicon wafer, using a photoresist technique developed in this study. Figure 26 (b) and (c) show the same pattern after expansion. The diameters of the unexpanded microspheres are 10-16 µm. The shown pattern is a test pattern consisting of 100 µm wide parallel lines with vertical 15 µm wide lines with a pitch of 100 µm. The density of deposited microspheres, *i.e.* the number of microspheres per area, can be influenced by varying the amount of microspheres per photoresist volume or by varying the spin speed.

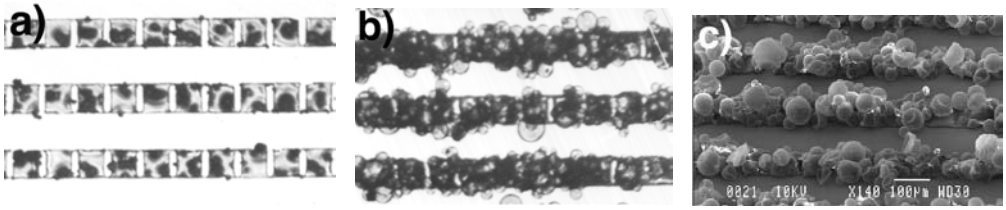


Figure 26. Photos showing selectively deposited (using photoresist) expandable microspheres before (a) and after (b) and (c) expansion. The horizontal lines are 100 µm wide and the vertical short lines are 15 µm wide.

Future applications of the expandable microspheres can be to replace fluidic components such as one-shot valves or micropumps. The expandable microspheres might also be used to coat the internal walls of microfluidic channels to enlarge the reactive surface area. It should also be possible to chemically modify the surface groups on the microspheres in a variety of ways for screening and analysis within the fields of biochemistry and organic chemistry.

3.3 Summary and Outlook

The miniaturization of analytical systems is progressing at a rapid rate, surface-based microarray systems are already invaluable in the field of genetic analysis and microfluidic systems are starting to appear on the market.

Until today, most microfluidic systems which have been presented are good demonstration vehicles for the feasibility of molecular assays in microscale, but their practical implementation for low-cost applications presents more problems. True lab-on-a-chip devices should offer the possibility of reducing costs by integrating processes that would otherwise require a suite of instruments and several manual manipulations in which human error and contamination may be introduced. For example, a system that demonstrates the potential of integration was constructed for the analysis of nanoliter volumes of DNA [89]. The device is capable of DNA amplification, or digestion, and labeling and separation of the resulting products. This compact system (47 mm long, 5 mm wide and 1 mm high) incorporates fluidics, pressure mobilization, controlled-temperature reaction chambers, sensors, gel electrophoresis and a fluorescence diode detector in a single unit, see figure 27. This is one of the first systems reported that approaches a fully integrated lab-on-a-chip for DNA analysis. In the future we will hopefully see lab-on-chips on the market which have the whole sequence from sample collection to analysis integrated on one chip.

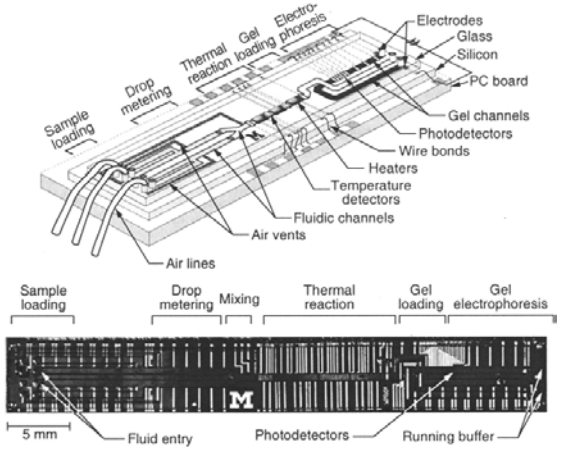


Figure 27. Schematic and photograph of an integrated device with nanoliter liquid injector, a sample mixing and positioning system, a temperature controlled reaction chamber, an electrophoretic separation system and fluorescence detectors. Reproduced from [89] with permission.

4. Microfluidic Devices for Organic Chemical Applications

In chapter 3 miniaturized systems for chemical analysis are described as a rapidly developing research area, encompassing chemical and biological applications. Commercial systems are already available, most notable for DNA analysis. However, the application of microfabricated devices to synthetic applications is deriving similar interest [90-91]. Microfluidic devices for synthetic applications are commonly called microreactors. The advantages conferred by microfluidic systems that are summarized in table 1 also apply to microreactors. In addition, when working with organic chemistry, the small scales used reduce exposure to toxic or hazardous materials. The greatest contribution to safety is the fact that hazardous materials can be synthesized as required at the point of use, in precisely defined quantities, thus eliminating the problems associated with transportation and storage.

Potential applications of microreactors include highly exothermic reactions *e.g.* the direct fluorination of aromatic compounds, the *in situ* generation of hazardous compounds, *e.g.* phosgene, and rapid energy transfer systems, *e.g.* fuel pumps [90-91]. Microreactors have so far been successfully deployed in gas and liquid phase chemistry, including catalyst testing [92-94].

The following section will focus on the development of microfluidic devices for liquid phase catalyst screening.

4.1 Devices for On-Chip Catalysis

On-chip heterogeneous catalysis represents an important area in organic synthesis and there are clear benefits in developing miniaturized synthetic reactors utilizing minimum amounts of catalyst in conjunction with very limited reaction volumes. Miniaturized catalytic devices can also be used to conveniently gain kinetic and thermodynamic data for reactions, as well as for screening new reactions and catalysts. Microreactors using heterogeneous catalysts have been applied in liquid-phase organic synthesis. An early comparatively recent development was reported from the Micro Reactor Group in Hull [95]. The microreactor utilized electroosmotic flow to mobilize the reagents and allowed synthesis of 4-cyanobiphenyl using a modified catalytic Suzuki coupling reaction. The incorporation of micro porous silica frits [96] within the reactor manifold enhanced the electroosmotic flow and allowed the immobilization of the heterogeneous catalyst (palladium). The synthesis of 4-cyanobiphenyl was achieved at room temperature, via *in situ* generation of base giving a product yield of about 67%. Conventional laboratory batch methodology using the same reaction criteria as used with the microreactor was performed resulting in a non-optimized product of 10%. Further work is currently

proceeding to improve the product yield in the microreactor by developing a post-reaction separation system, which allows the recycling of starting material and the isolation of a pure product.

The Hull group has also demonstrated that a superacid catalyst (sulfated zirconia) could be immobilized onto the surface of a PDMS microreactor top plate [97]. This was achieved by dusting the pre-cured PDMS surface with activated catalyst before baking the plate. The top plate was clamped to a glass base plate with etched channels and a syringe pump was used to mobilize hexan-1-ol, which thereby underwent dehydration to hex-1-ene. The microreactor featured an *in situ* resistive heater wire cast into the PDMS top plate, which was operated at about 160 °C. The conversion efficiency was reported to approach 100% which can be compared with the industrial process that only gives approximately 30% conversion.

A borosilicate microreactor that allowed the synthesis of a number of nitrostilbene esters has been reported [98]. This microreactor enabled investigation of the Wittig reaction by studying a number of reaction features such as stoichiometry, stereochemistry and reaction diversity. This work has also been extended to investigate the stereoselective control of the chemical reaction by applying electrical fields which generated controlled concentration gradients of the reagent streams [99].

Asymmetric catalysis is a powerful technique used *e.g.* by the pharmaceutical industry in the search for new active compounds for pharmaceuticals. In this thesis, ligands for asymmetric catalysis have been immobilized directly on deep reactive ion etched channel walls by consecutive microcontact printing (Paper 6). The technique involves two subsequent printing steps using unstructured PDMS stamps. The pattern is already defined on the substrate, consisting of etched channels, see figure 28. Hence, no precise alignment is needed between the two printing steps.

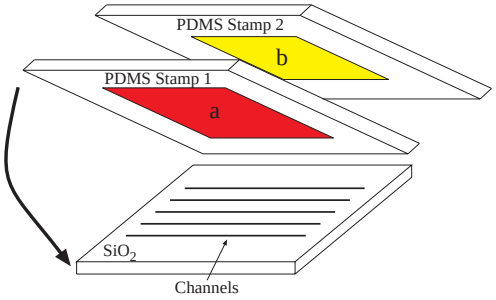


Figure 28. A schematic showing the principle consecutive microcontact printing in channels, where a and b represent different reagents.

Pybox (2.6-bis(oxazolyl)pyridine) which is a chiral ligand capable of performing asymmetric catalysis, was immobilized using consecutive microcontact printing (Paper 6) according to the scheme presented in figure 29. The synthetic procedure involved the transformation of the surface via μCP of 4-(chlorosulfonyl)benzoic acid, anhydride formation (in solution) and finally μCP of the ligand (2,6-bis(oxazolyl)pyridine).

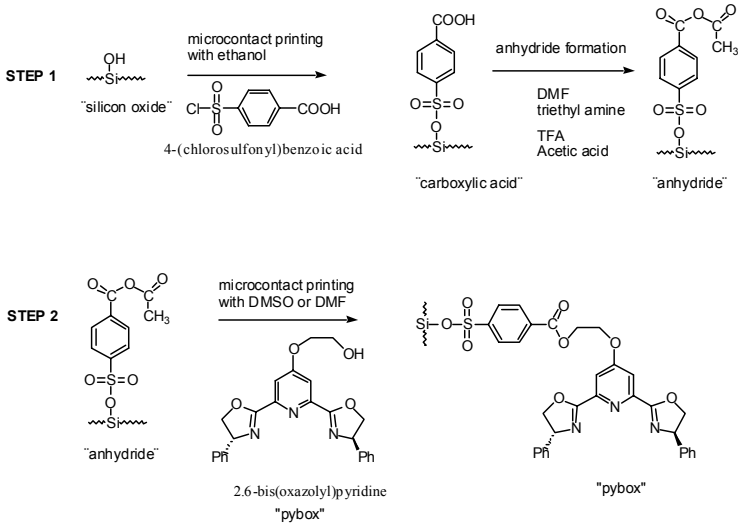


Figure 29. Synthetic scheme of consecutive microcontact printing of 4-(chlorosulfonyl)benzoic acid and 2,6-bis(oxazolyl)pyridine.

The molecular layers formed were evaluated by contact angle measurements, SEM and electron spectroscopy for chemical analysis (ESCA), and indicated that the consecutive microcontact printing was successful. The channels were sealed with a PDMS film, using a low temperature bonding technique which does not destroy the printed ligand.

The activity of the immobilized ligands is currently being investigated, as well as the downscaling of the reaction protocols. A possible chip design for performing combinatorial assays with the immobilized catalysts would be an array of channels or reaction chambers with common or separate inlets and outlets, as illustrated in figure 30, where the catalysts are attached on the internal walls. In case the amount of immobilized catalyst in the channels is insufficient, the consecutive microcontact technique will be applied to channels of porous silicon instead. Porous silicon has larger surface area and has previously been used as a coupling matrix for enzyme coupling [100].

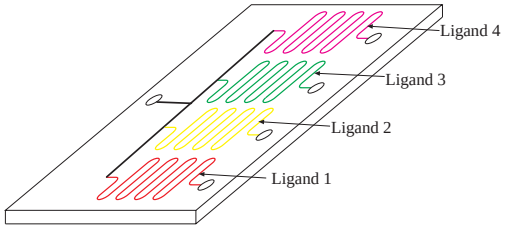


Figure 30. A conceptual drawing of an array of flow-through serpentine channels for combinatorial assays.

An alternative way to increase the surface area and allow greater flexibility, *i.e.* rendering the system more gel-like, is to use a polymer as a linker. In paper 7 and 8 it has been shown that polymer beads with different functionalizations can be self-assembled and immobilized in monolayers on unstructured and structured silicon. Methods for functionalization of polymers with biomolecules, catalysts, reagents etc. are well known [101]. Therefore, it would be rather straightforward to further functionalize the immobilized polymer beads with the desired ligand, for example. These platforms are currently being further developed to be applied in catalyst screening.

If a monolayer of beads does not provide enough active surface area for certain synthetic applications the filter-chamber presented in paper 1 might be useful. In this device the beads can be packed in three-dimensions, hence a larger number of beads can be held using the same chip area. The suitability of the filter-chamber device in catalyst screening is currently being evaluated.

4.2 Summary and Outlook

The examples given above show that microreactors can bring novelty and real practical advantages to reaction-based chemistry. The advantages come essentially from the thermal, spatial, and temporal control possible in such devices, coupled with the capability to monitor reactions *in situ* while operating. Work is currently underway that will rapidly push the technology towards working devices for combinatorial and controlled multi-stage syntheses. Issues over product volumes will always be raised when using microreactors but given the inherent practical advantages of the methodology, these will already be attracting the necessary engineering and design developments to realize appropriate system scale-out.

A commercially available chemical synthesizer using microreaction technology already exists, and is produced by the Institute of Microtechnology Mainz (IMM), Germany, see figure 31. It consists of a pumping module, a microreactor that results in efficient mixing of reagents, followed by a capillary to allow time for the reaction to go to completion. The outflow is then collected for further manipulation by the user. This could be the first step along the road which will see the integration of automated reagent manipulation, reaction monitoring, and product purification into a single instrument.

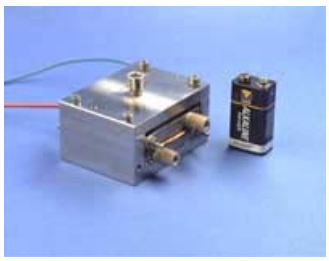


Figure 31. A microreactor for electro-synthesis of organic components developed by IMM, Germany (http://www.imm-mainz.de).

5. Summary of Appended Papers

The appended papers describe the design, fabrication, and evaluations of different microfluidic devices for biotechnical and organic chemical applications. The papers are presented in chronological order. Paper 1 presents the design, fabrication and fluid characteristics of the filter-chamber. Paper 2 presents hydrophobic valves in DRIE channels and their suitability for biochemical applications. Paper 3 presents filter-chamber arrays with hydrophobic valves. Paper 4 presents an investigation about the suitability of valve-less micropumps for biochemical applications. Paper 5 presents a low temperature adhesive bonding technique for structured wafers. In paper 6 the immobilization of asymmetric ligands using consecutive microcontact printing is described. Paper 7 and 8 present self-assembly and self-sorting of beads based on surface chemistry on unstructured and structured substrates. In paper 9 SNP analysis in the filter-chamber is presented. Paper 10 presents expandable microspheres as a novel tool in microfluidics.

Paper 1

A new flow-through micromachined device for chemical reactions on beads has been designed, manufactured, and characterized. The device has an uncomplicated planar design and microfabrication process. Both nonmagnetic and magnetic beads can be collected in the reaction chamber without the use of external magnets. The device is sealed with Pyrex to allow real time optical detection of the chemical reactions. At a constant pressure of 3 kPa at the inlet the flow rate for water is about 3.5 µl/min without beads in the filter chamber. The smallest reaction chamber has a volume of 0.5 nl and can collect approximately 5740 beads with a diameter of 5.50 µm. The flow rate decreases by about 40% when the reaction chamber is packed with beads. The flow-through microfluidic device is not sensitive to gas bubbles, and clogging of the filter is rare and reversible. The beads are easy to remove from the reaction chamber making the micromachined flow-through device reusable.

Paper 2

The suitability of using octafluorocyclobutane (C_4F_8) patches as hydrophobic valves in microfluidic biochemical applications is presented. A technique has been developed to generate lithographically defined C_4F_8 hydrophobic patches in deep reactive ion etched silicon channels. Different patch lengths (200-1000 µm) of C_4F_8 were deposited in 50 µm wide channels to evaluate which size is most suitable for microfluidic biochemical applications. The valve has a high chemical resistance to a wide variety of liquids, aqueous and non-aqueous, relevant in biochemistry including solutions of microbeads. Patch

lengths of 200 µm of C_4F_8 successfully stopped each solution for at least 20 consecutive times. The hydrophobic valve also resists very high concentrations (25%) of surfactants. C_4F_8 shows a much higher resistance towards water and surface active solutions than previous hydrophobic patches. An applied pressure of 760 Pa at the inlet was needed for water to over run the hydrophobic patch.

Paper 3

The filter-chamber array presented here enables real-time parallel analysis of three different samples on beads in a volume of 3 nl, on a 1 cm^2 chip. The filter-chamber array is a system, containing three filter-chambers, three passive valves at the inlet channels and a common outlet. The design enables parallel sample handling and time controlled analysis. The device is microfabricated in silicon and sealed with a Pyrex lid to enable real time analysis. The passive valves consist of plasma deposited octafluorocyclobutane as presented in Paper 2. The device is not sensitive to gas bubbles, clogging is rare and reversible, and the filter-chamber array is reusable. More complex (bio)chemical reactions on beads can be performed in the devices with passive valves than in the devices without valves.

Paper 4

The suitability of valve-less micropumps in biochemistry is presented. Fluids encountered in various biochemical methods that are problematic for other micropumps have been pumped with good performance. The pump is fabricated as a silicon-glass stack with a new process involving three subsequent deep reactive ion etching steps. The micropump is self-priming and insensitive to particles and bubbles present in the pumped media. The results show that the valve-less micropump successfully pumps fluids within the viscosity range 0.001-0.9 Ns/m^2. The micropump is not sensitive to the density, ionic strength, or pH of the pumped media. Effective pumping of solutions containing beads of different sizes was also demonstrated. Living cells were pumped without inducing cell damage and no cell adhesion within the pump chamber was found. No valve-less micropump has previously been reported to pump such a wide variety of fluids.

Paper 5

A technology for void free low temperature full wafer adhesive bonding of structured wafers is presented. Benzocyclobutene (BCB) is used as the intermediate bonding material. BCB bonds well with various materials and does not release significant amounts of by-products during the curing process. Thus void-free bond interfaces can be achieved. Bonding of fluidic structures and the protective sealing of protruding structures on a wafer surface is presented. An important finding is that the pre-cured BCB coatings are extremely deformable and have a "liquid-like" behaviour during bonding.

Paper 6

Consecutive microcontact printing has been developed to enable multiple functionalization of silicon surfaces, such as the immobilization of chiral ligands. The technique involves two subsequent printing steps using unstructured poly(dimethylsiloxane) stamps. The pattern is already defined on the substrate, consisting of etched channels. Hence, no precise alignment is needed between the two printing steps. A carboxylic acid group-containing reagent was initially printed onto the silicon dioxide surface and transformed to an anhydride. In the second printing step an ester bond was formed with the hydroxy-functionalized ligand. The formed molecular layers were evaluated by contact angle measurements, SEM and ESCA indicating that the consecutive microcontact printing was successful.

Paper 7

A technique for generating a general screening platform consisting of dots of immobilized beads on silicon has been developed via self-sorting and self-assembly of different kinds of beads. The dots are defined by a teflon-like film, which due to its hydrophobic characteristics also prevents cross-contamination of liquid from different dots. To enable functionalization of individual dots with different target molecules simultaneously a new way of microcontact printing has been explored where different target solutions are printed in parallel using one stamp. To show that this platform can be designed for both biochemical assays and organic chemistry, streptavidin-, amino- and hydroxy-functionalized beads have been self-sorted and self-assembled both on separate and common platforms. Beads of different sizes and material have successfully been immobilized in line patterns as narrow as 5 μm. Besides silicon, quartz and polyethylene have also been used as substrates.

Paper 8

A novel technique enabling selective bead trapping in microfluidic devices without the use of physical barriers is presented in this paper. It is a fast, convenient and simple method, involving microcontact printing and self-assembly, that can be applied to silicon, quartz or plastic substrates. In the first step, channels are etched in the substrate. The surface chemistry of the internal walls of the channels is then modified by microcontact printing. The chip is submerged in a bead slurry where beads self-assemble based on surface chemistry and immobilize on the internal walls of the channels. Silicon channels (100 µm wide and 50 µm deep) have been covered with monolayers of streptavidin-, amino- and hydroxy-functionalized microspheres and resulted in good surface coverage of beads on the channel walls. A high-resolution pattern of lines of self-assembled streptavidin beads, as narrow as 5 µm, has also been generated on the bottom of a 500 µm wide and 50 µm deep channel. Flow tests were performed in sealed channels with the different immobilized beads to confirm that the immobilized beads could withstand the forces generated by water flowing in the channels. The presented results indicate that single beads can be precisely positioned within microfluidic devices based on self-assembly which is useful as screening and analysis tools within the field of biochemistry and organic chemistry.

Paper 9

In this paper the principle of allele-specific extension using pyrosequencing chemistry was employed to analyze single nucleotide polymorphism (SNP) in the filter-chamber device presented in paper 1. Two SNP sites located on chromosome 17p (codon 72 of the p53 gene) and 9q (wiaf 1764) were selected to evaluate the device. Single-stranded target DNA was obtained by using nonmagnetic streptavidin-coated beads. Primers with alternating 3'-ends were hybridized to the target DNA and were captured in the filter-chamber of the flow-through device. Pyrosequencing reagents including all four nucleotides were applied and an external CCD camera detected the produced light. The bead-trapping device enabled analysis of genetic variations by using pyrosequencing chemistry in reaction chamber volume as small as 12.5 nL and the results demonstrate the possibility of performing SNP analysis by this technique in small volumes.

Paper 10

In this paper a novel component for microfluidics is introduced. Expandable microspheres have been studied for their application in microfluidics. Two methods for selective immobilization of expandable microspheres on silicon, including patterning by photolithography and self-assembly based on surface-chemistry has been shown. After the immobilization step the microspheres were expanded thermally. The expansion is irreversible and the volume of the microspheres increases more than 60 times. Patterns of microspheres with features as small as 15 μm have successfully been generated by photolithography. By using self-assembly the microspheres can conveniently be immobilized in a monolayer.

6. Conclusions

The main objective of this thesis research, which was to develop novel microfabrication techniques and microfluidic devices for applications in the fields of biotechnology and organic chemistry, has been fulfilled. However, further evaluation of the devices for the specific applications is needed and might result in changes in the designs. Among the important results from this broad objective are the following:

- A filter-chamber device with passive valves has been designed, fabricated and evaluated for DNA analysis using pyrosequencing. The fabrication process is simple and involves clever use of the deep reactive ion etching machine. The reactive surface area consists of mobile beads which easily can be modified and exchanged. The device is reusable and suited for parallelization. The hydrophobic valves function well for biochemicals and beads. It was demonstrated that the filter-chamber device enables typing of SNPs by allele-specific extensions in a volume of 12.5 nl which is a 4 000 fold reduction compared to the standard volume.

- Microcontact printing has been utilized for surface modifications in a novel manner in this thesis work. Ligands for asymmetric catalysis have been immobilized in deep reactive ion etched channels using microcontact printing. The printing was performed in a consecutive manner using unstructured stamps and structured substrates. In this way, there is no need for alignment between the printing steps. The feasibility of performing asymmetric catalysis reactions in these channels is currently being investigated.

- A technique for generating a general screening platform consisting of dots of immobilized beads on silicon has been developed via self-sorting and self-assembly of different kinds of beads. In order to show that this platform can be designed for both biochemical assays and organic chemistry, streptavidin-, amino- and hydroxy-functionalized beads have been self-sorted and self-assembled both on separate and common platforms. The self-sorting and self-arrangement are based on surface chemistry only. The feasibility of performing pyrosequencing on this surface-based system of immobilized monolayers of beads is currently being investigated.

- A novel technique enabling selective bead trapping in microfluidic devices without the use of physical barriers has been developed. It is a fast, convenient and simple method, involving microcontact printing and self-assembly. Etched channels can be patterned with monolayers of streptavidin-, amino- and hydroxy-functionalized microspheres with good surface coverage of beads even on the channel walls. The presented results indicate that single beads can be precisely positioned within microfluidic devices based on self-assembly. The feasibility of performing asymmetric catalysis reactions and pyrosequencing in this flow-through system of immobilized monolayers of beads is currently being investigated.

- Expandable microspheres have been introduced as a novel tool in microfluidics. Expandable microspheres are small spherical plastic particles which irreversibly increase their volume upon heating. Two methods for selective immobilization of expandable microspheres on silicon, including patterning by photolithography and self-assembly based on surface-chemistry has been shown. Future applications of the expandable microspheres may be to replace fluidic components such as one-shot valves or micropumps.

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