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Del Bueno, Nadia

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**Bioanalytical Microfluidic Devices and Methods for
Analysis of Cancer Gene Expression**

by

Nadia Del Bueno

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Joint Doctor of Philosophy

with University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Richard A. Mathies, Chair

Professor George F. Sensabaugh

Professor John Kurhanewicz

Spring 2011

Bioanalytical Microfluidic Devices and Methods for Analysis of Cancer Gene Expression

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By Nadia Del Bueno

Abstract

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Nadia Del Bueno

Joint Doctor of Philosophy in Bioengineering

University of California, Berkeley

and

University of California, San Francisco

Professor Richard A. Mathies, Chair

Transcriptional profiling is essential in fundamental studies of pathogenesis. In particular, multiplexed analysis of gene expression enables the identification of cancer-specific expression signatures that correlate with clinical disease and which can be used for the prediction of tumor presence and disease progression. Microfabricated capillary electrophoresis (CE) devices feature reduced sample and reagent requirements, faster analysis times, and increased automation. These advantages make microdevices ideal analytical platforms for the quantitative monitoring of biomarkers, and their potential as point-of-care devices for facilitating cancer detection in the clinical setting is explored in this thesis.

First, an integrated microdevice capable of performing low-volume, rapid, and highly sensitive expression analysis was developed. To further the goal of quantitative measurements of transcript levels from a small amount of sample, an affinity capture gel approach was used to address the problem of inefficient CE sample injection which limits sensitivity. Photopolymerization protocols were developed to define a small plug of oligonucleotide-modified polyacrylamide gel inline with the CE channel in order to accomplish efficient capture and sample microinjection for quantitative analysis. This concentration and purification step also demonstrated increased detection sensitivity and improved separation resolution.

To perform multiplexed analysis of cancer genes, an optimized protocol for transcript analysis with aid of affinity capture was used in conjunction with the integrated CE microdevice. The expression of genes implicated in prostate cancer was assayed directly from cells by solution hybridization with complementary fluorescently-labeled detection probes, followed by affinity bead capture of mRNA-probe complexes. Released detection probes were then injected on-chip and captured on a photopolymerized capture gel for concentration prior to CE separation and detection. The ability of the assay and microdevice

system to evaluate gene expression was demonstrated by the measurement of absolute transcript levels of ten genes, enabling the successful identification of distinct expression signatures for the human prostate cancer cell lines LNCaP, VCaP, 22Rv1, and PC-3 with high sensitivity.

Finally, a microdevice also including on-chip PCR amplification is presented for improving the sensitivity of detection to enable the analysis of clinical samples. The functionality of the proposed microsystem is further expanded by the integration of upstream sample processing steps on-chip. By performing several bioanalytical processes on a single microfabricated platform, high-sensitivity expression analysis from complex biological samples should be possible, all the while reducing cost and analysis time. Based on the technologies developed in this thesis, these fully integrated devices could be implemented as diagnostic tools and play a key role in the future of clinical detection.

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Chapter 1

Gene Expression Methods for Cancer Pathology

1.1 Motivation

1.1.1 Need for Cancer Detection Methods

Cancer is a frustrating disease to study. Evidence of the disease exists even in the earliest records of human history, yet it is still responsible for over 560,000 deaths per year in the United States alone [1]. The goal of a cancer cure remains a focus of biomedical research, funded both by private endeavors and by the National Institutes of Health (NIH) to the tune of over \$5.8 billion a year [2]. Thanks to these efforts, there has been some success in the fight against this complex disease. Notably, the number of cancer deaths has dropped, even as the rate of incidence of new cases has increased [1]. Over 200 forms of human cancer have been documented so far, each characterized by uncontrolled cell growth, invasion to adjacent tissues, and in the worst cases, metastasis. Clearly, further research is needed, as much remains to be learned about the causes and treatment of this devastating disease. In particular, continued success in developing new cures necessitates improvements in our knowledge of the fundamental mechanisms of cancer, as well as in our ability to accurately detect and characterize the disease.

Whereas several cases of cancer had been described by Hippocrates' lifetime, it was not until the nineteenth century that the first true attempts at a scientifically rigorous identification of the origins of the disease began. Johannes Müller established in 1838 that cancer was a collection of diseased cells [3]. However, the causes of the disease and mechanisms of tumor development remained a mystery. The genetic basis of cancer was finally recognized in 1902 by Theodor Boveri, a German zoologist who postulated that the function of chromosomes was to transmit various inheritance factors [4]. He also suggested that alterations in these chromosomes could lead cells to divide uncontrollably, and correctly identified this unlimited growth potential as the source of cancerous tumors. He further hypothesized that cancers could be caused by radiation and exposure to chemical agents as well as pathogens.

Our current understanding of the genetic causes and biochemical processes involved in cancer really took off with the discovery of the double helix structure of DNA by Watson and Crick in 1953, as the description of base pairing rules revealed how genetic information is conserved and inherited. An explosion in technological development around this newfound understanding of DNA led to the discovery of several novel techniques that facilitated the manipulation of biological molecules, including polymerase chain reaction (PCR) [5], molecular cloning [6], and Sanger sequencing [7]. This in turn allowed the identification of critical genes, such as those implicated in the cancer proliferation and invasion pathways. Researchers quickly realized the link between mutations in these genes and abnormal growth, and tumor-specific genes became the central focus of several studies with a goal of finding new therapies by developing biochemical agents aimed at these molecular targets [8,9]. At the same time, the identification of "high-risk genotypes" has allowed the articulation of preventative measures to benefit patients who are genetically predisposed to certain cancers [10,11]. However, more than 90% of cancers do not have a hereditary genetic cause, and understanding of the fundamental biological pathways involved is still lacking for most cancers. Developing new methods to detect various types of cancer is especially important as it expands our ability to diagnose the disease early and broadens treatment options.

1.1.2 Gene Expression Analysis

Two classes of genes play a crucial role in cancer progression by controlling cell life span, growth, and division. Oncogenes have the potential to cause cancer by interfering with apoptosis, thereby causing cells to survive and proliferate instead of undergoing programmed cell death. Proto-oncogenes are a subcategory that consists of naturally occurring genes which are capable of becoming oncogenes due to mutations or increased expression. Because the natural function of these genes is to regulate cell growth and division, they can promote tumor growth through overexpression. However, most oncogenes do not cause cancer until the mutation of additional genes or exacerbation by environmental factors. In contrast, tumor suppressor genes promote tumor growth when they are under-expressed or diminished in activity by mutations. TP53, a gene frequently altered in human cancers belongs to this class [12]. The TP53 gene encodes for tumor suppressor protein p53, which regulates the cell cycle and therefore prevents cancer. Loss of function of this gene results in uncontrolled cell growth and is a common factor observed in many types of cancer.

The number of genes implicated in cancer is considerable, and the role of each is highly variable based on the type of cancer and disease stage. Over the past three decades, cancer research has benefited from key advances in the identification of individual genes and molecules with potential roles in disease progression. More recently, the wealth of genomic information provided by the Human Genome Project has shifted the focus of biological investigation to genome wide profiling. This has often led to the identification of genes that play a major role in their specific cancer type. For example, the discovery of BRCA genes has led to the stratification of breast cancer types and the development of genetic tests for the detection of high-risk mutations to guide treatment [13,14]. Similarly, numerous essential prostate genes have been identified, including genes implicated in prostate cancer such as PSA [15], PCA3 [16], and more recently, AMACR [17]. However, our understanding of the genes involved in cancer remains incomplete, and further work is necessary to reveal the extensive pathways that characterize tumor progression.

While direct genomic sequencing helps researchers discern mutations, which are the basis of many types of cancer, profiling of gene expression levels is essential in fundamental studies of pathogenesis. Expression analysis of genetic targets reveals how the gene transcripts are interpreted and synthesized into the products that give rise to the cancer phenotype. Specifically, the analysis of expression of tissue and cancer-specific genes enables the identification of expression patterns that can be monitored during normal and disease states, in this manner providing valuable information for understanding the complex interaction of individual genes in cancer progression. Moreover, multiplexing the number of biomarkers analyzed can overcome the limitations of single genetic marker assays, which cannot reliably be used alone for cancer detection. For example, serum prostate-specific antigen (PSA) is used extensively for screening [18] but suffers from substantial limitations, including a lack of prostate cancer sensitivity and specificity. In contrast, multiplexed expression analysis can be used for the identification and characterization of cancer specific expression signatures for the prediction of tumor presence and progression [19,20]. In addition, stratification between clinically important and clinically insignificant forms of cancer can be made possible by monitoring specific prognostic markers. This can supplement histological examination in predicting metastasis and clinical outcome, thus

enabling customization of therapeutic options as well as monitoring of response to specific treatments. Besides facilitating diagnosis and treatment, the benefits of gene expression analysis and the identification of expression fingerprints implicated in cancer pathogenesis are certainly relevant to all areas of cancer research.

1.1.3 The Advantages of Studying RNA

Because of its role as the genetic carrier of information, DNA is often considered the most important biological molecule. However, the Central Dogma of molecular biology, formulated in 1958, drew attention to the central function of RNA by proposing that genetic information is transcribed from a DNA template to RNA and then translated from RNA into proteins. Under this concept, RNA molecules carry the genetic information stored in the DNA and direct the synthesis of the proteins necessary for cell function. Analyzing RNA expression therefore bridges the link between the genetic basis of cancer and the proteins that cause the chemical changes in the cell. The importance of RNA has even led to the formulation of the “RNA world” hypothesis, which proposes that life based on RNA predates current life based on DNA and proteins [21]. This premise is supported by RNA’s unique ability to perform functions of DNA as well as those of proteins: like DNA, RNA can store, transmit and duplicate genetic information, but it can also behave like a protein, for example by having enzymatic activity [22].

The chemical composition and structure of RNA, while seemingly only different from those of DNA in a minor way, enable this multi-functionality. Like DNA, RNA is made up of a long chain of nucleotides, arranged in a sequence of three base codons, which allow RNA to encode genetic information. The major difference is that unlike DNA, RNA is single stranded, and employs the uracil (U) rather than the thymine (T) nucleotide. Structural analysis of RNA reveals a complex organization that makes RNA a versatile molecule capable of playing many roles in the biology of the cell. RNA molecules do not consist of the long double helices that have become identifiable features of DNA. Instead, RNA single strands fold and hybridize within themselves, producing repeating structures composed of beta sheets and short hairpin double helices packed together.

These differences create some challenges when working with RNA. First, because RNA contains a 2-hydroxyl in its ribose backbone rather than a 2-deoxyribose group, the molecule is very susceptible to degradation by hydrolysis. As a result, working with RNA necessitates meticulous protocols to prevent sample degradation. Second, RNA takes many forms in the cell: messenger RNA (mRNA) encodes the genetic information for the corresponding protein product, while transfer RNA (tRNA) mediates recognition of the codon, and ribosomal RNA (rRNA) plays a part in the protein manufacturing process in the ribosome. Studies of gene expression are mainly interested in mRNA molecules, however these account for only 1-5% of total RNA samples while rRNA, mostly in the form of the 28S and 18S rRNA species, makes up over 80%. Because of this, detecting mRNA for expression analysis is challenging even with the most sensitive methods.

Nevertheless, using RNA is the best way to study changes in gene expression to shed light on complex biomolecular mechanisms. Because RNA is able to undergo rapid modifications through mechanisms of editing, alternative splicing and silencing, the manner in which the RNA is expressed is often more revealing than the presence of the gene itself. An alternative is to analyze the cell’s protein content directly, which is the optimal approach

for measuring expression since RNA does not always accurately represent cellular protein content. This is because measuring RNA does not take into account post-transcriptional modifications of RNA or post-translational modifications of the protein, both of which play a crucial role in the protein's functionality. Whereas proteomic analysis has been used for finding consistent expression profiles in certain cancers [23,24], analyzing proteins directly remains problematic. One challenge is that proteins vary largely in the number of copies of each molecule, with most proteins well below the limits of detection offered by standard laboratory methods. In addition, unlike for nucleic acids, there are no existing methods for performing amplification of proteins. Another difficulty is the lack of good techniques for measuring cellular protein content. Green fluorescence protein (GFP) can be used to directly measure a cell's expression of a particular protein by coupling a GFP reporter to a protein target and visualizing its fluorescence. However, the method is laborious and is limited by spectral overlap to the detection of a few proteins. Due to the complex shapes and varying charges of proteins, electrophoretic separations are also more challenging than for RNA and necessitate two-dimensional electrophoresis where isoelectric focusing is used along with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to achieve separation. Because RNA is more easily amplified and manipulated than proteins, it remains a better way to perform gene expression analysis.

1.2 Methods of RNA Analysis

The measurement of RNA expression levels is essential in studies of pathogenesis as well as for the detection and monitoring of gene expression fingerprints implicated in cancer for diagnostic purposes. Accordingly, there has been a growing demand for novel methods that allow quantification of gene expression. This measurement of RNA must overcome several challenges, including the low concentration of mRNA in most samples, the easy degradation of RNA molecules, as well as the large variations in the abundance of transcripts which can range from thousands of copies per cell to a single copy. Ideally, the analytical method for measuring expression must be robust and enable fast, sensitive and quantitative monitoring of mRNA levels from a small amount of sample. The most commonly used techniques for gene expression analysis include Northern blots, microarray analysis, serial analysis of gene expression (SAGE), and real-time PCR (qPCR).

1.2.1 Northern Blots

Northern blotting, developed in 1977 by Alwine et al. [25] has become a common method for molecular biologists to study gene expression by measuring the RNA content of a sample. For example, the method has been used successfully for comparing patterns of gene expression between different tissues. Of specific importance for cancer, Northern blots have been used to demonstrate oncogene overexpression and tumor-suppressor gene downregulation in cancer cells compared to normal cells [26]. In fact, so many expression studies have been done using Northern blots that a database called BlotBase, has been created to serve as a repository of over 700 published Northern blots spanning hundreds of genes and many tissue types [27].

The widespread use of Northern blots for gene expression studies in so many applications can be attributed to the simplicity of the protocol. The analysis begins with the extraction of total RNA from a homogenized lysate of cells. Then, RNA is separated based on size by electrophoresis, using agarose or polyacrylamide denaturing gels that contain either formaldehyde or urea to limit secondary structures. The sizes of the fragments obtained are determined during electrophoresis of total RNA samples simply by comparing to the ribosomal subunits that can serve as size markers: the 28S large ribosomal subunit, about twice the size of the 18S small ribosomal subunit, yields a band about twice as intense. After separation, RNA samples are transferred to a positively charged nylon membrane which has high affinity for the negatively charged RNA. Finally, labeled probes are hybridized to the immobilized RNA, and hybrids thus formed are detected.

A key advantage of Northern Blots is that they enable direct detection of RNA, which results in the unbiased analysis and quantification of the sample. However, the method suffers from several disadvantages. The protocol necessitates many steps, including several sample transfers which cannot easily be automated. In addition to being time consuming, this extensive RNA handling requires meticulous technique to prevent sample degradation by ubiquitous RNAses. In addition, specificity and efficiency of the hybridization steps are highly dependent on experimental conditions, and lack of automation translates into diminished reproducibility of the results. Another drawback is that expression analysis by Northern blot requires samples containing a minimum of thousands of cells, and this lack of sensitivity means that genes with low copy numbers may not be detected. For

these reasons, Northern blotting is typically only feasible for the analysis of a handful of genes, and as such is currently mostly used for verifying expression results obtained by microarray analysis.

1.2.2 Microarrays

A microarray consists of a large array of microscopic oligonucleotide spots, each containing a short probe that hybridizes to a single gene target with high specificity. Since the demonstration in 1987 of the first DNA microarrays, created by pin-spotting of DNA onto filter paper [28], microarrays have evolved in design, fabrication, and costs. The Affymetrix microarray, first patented in 1992, uses covalent attachment to a solid surface [29]. Although the most commonly used substrate is glass, others such as silicon and plastics have also been demonstrated. Other microarray platforms, such as those commercialized by Illumina, replace the solid surface with microscopic polystyrene beads and are for this reason known as bead arrays [30]. Despite these differences, all the various types of microarrays share the same goal of enabling parallel and multiplex genetic analysis on a miniaturized platform and in a highly automated fashion.

Microarrays utilize the inherent ability of complementary nucleic acids to hybridize with each other in a specific manner with varying stringency depending on the number of complementary bases. Before analysis by microarray can begin, cells must be lysed and their mRNA extracted. Rather than using the RNA directly, mRNA is reverse transcribed into cDNA, labeled by coupling with Cy3 (green) or Cy5 (red) nucleotides, and finally hybridized to the microarray under binding conditions. Stringency washes are used to remove unhybridized and non-specific sequences. After hybridization, the array is scanned for visualization of the results: the intensity of each spot indicates the amount of target present in the sample, while the identity of the gene transcript is defined by its position on the array.

Because of the thousands of probes contained on each microarray, a high level of parallelism is possible, and a complete profile of expression can be obtained in one experiment. This has helped microarray analysis become standard in the study of expression since its first publication in 1994 [31,32]. Differences in expression profiles of human cancer compared to healthy samples were reported by 1999 [33], precipitating the identification of specific expression profiles for various cancers. Breast [34], ovarian [35], lung [36], and prostate cancer [37] have all been analyzed in this manner. The adoption of microarrays has further expanded as the costs of analysis have declined. Whereas the first gene chips were only accessible to a few researchers because of their high cost and the inability to reuse the device, inexpensive alternatives have since been developed [38,39]. The ability to print microarrays in house has further increased the level of customization and considerably reduced costs, since the number of probes can be narrowed to only those genes that are of interest.

Despite these advances, the method has some significant drawbacks. First, microarray analysis suffers from shortcomings in the results obtained. The large data sets collected from each experiment require significant analysis with specialized software to determine statistical relevance. More importantly, the results allow relative quantification only, as the transcript level of each gene can only be expressed as a ratio normalized to a control. It must also be noted that the expression results obtained assume that the cDNA

products of the reverse transcription accurately reflect the mRNA content of the original sample. This is not always the case, and this bias is further worsened by PCR amplification steps included in many microarray chips in order to decrease the required amounts of starting material necessary for analysis. This biasing of the transcription profile can especially be a problem for genes that were present in the sample at low copy numbers. Conversely, when PCR amplification is not used, the method lacks sensitivity. Generally, at least 5 μg of total RNA, or the equivalent of 2 to 5 million copies of each mRNA are necessary for detection by microarray without target amplification [40]. Because of the large amount of material necessary for expression analysis and the limited ability to quantify results, the usefulness of microarrays has so far been limited to research rather than diagnostic applications.

1.2.3 Serial Analysis of Gene Expression (SAGE)

Serial Analysis of Gene Expression (SAGE) is a method for performing quantitative gene expression analysis. Since its publication in 1995 by Velculescu, the technique has gained many followers as an alternative to microarrays when direct quantification of the results is desired [41]. While the method was originally used for cancer studies, its use has extended to other disease applications as well. The SAGE protocol is as follows: after isolating mRNA from the sample, a short sequence tag (~10-15 bp) is obtained from a defined position within each mRNA molecule. These sequence tags, which contain enough information to identify their corresponding transcripts, are then linked to form long chain molecules that can be cloned and subsequently sequenced using conventional DNA sequencers. The resulting data are processed to count the number of times each tag is detected, yielding information about the absolute level of expression of the original transcripts in the sample.

The technique is relatively straightforward and only requires standard molecular biology protocols. Since there are no hybridization steps, the results are more quantitative than those obtained by microarray analysis. In addition, another advantage of SAGE is the lack of bias in the resulting expression profile. Because oligo-dT is used for cDNA synthesis, reverse transcription of the mRNA molecules is sequence independent. Likewise, the use of universal primers for PCR leads to unbiased amplification of the tags and ensures that the results accurately reflect the gene transcript in the sample. Moreover, this sequence independent transcription and amplification allows researchers to use SAGE to quantify expression without prior knowledge of the transcripts, and SAGE has in this manner enabled the identification of several novel genes and new gene variants.

However, the method suffers from some drawbacks which have prevented it from becoming more widely adopted. First, SAGE is characterized by poor sensitivity and necessitates a large amount of template material; a minimum of 2 μg of poly-A mRNA or 2 million cells are usually required to perform SAGE. In addition, the protocol includes many time consuming steps necessary for processing the template in order to get the expression results. Finally, microarrays are a cheaper alternative, especially for performing large scale studies. Consequently, SAGE is usually reserved only for those cases when more exact quantification of transcripts is needed.

1.2.4 Real Time-PCR

Real time PCR or quantitative PCR (qPCR) is used to amplify a DNA template while simultaneously quantifying its expression after each PCR cycle. When performing PCR, RNA sample is first reverse transcribed to cDNA, after which the amplification of template enables the analysis of gene expression even from small amounts of sample. Conventional PCR necessitates a detection and quantification step after the end of the amplification cycle. In contrast, qPCR provides real time fluorescence detection of the DNA as the amplification progresses. The change in fluorescence intensity as the reaction proceeds indicates the amplification yield and enables quantification. A popular method of detection is to use SYBR green intercalating dye to label DNA: when the dye binds to the double stranded PCR product, it fluoresces with an intensity that is proportional to the number of amplicons generated. Because the dye intercalates all double stranded products in a non-specific manner, the quantification is not always accurate and is highly susceptible to contamination. An alternative method is the use of gene-specific reporter probes. These oligonucleotide probes are dual labeled with a fluorescent reporter at the 5' end and a fluorescent quencher at the 3' end [42]. Due to the close proximity of the quencher and the reporter, the probe is not detected until the fluorescent reporter is released by the activity of the Taq polymerase during the extension step of PCR.

When used with reporter probes, qPCR is characterized by high specificity. Quantification is also highly accurate, since non-specific DNA amplification is not detected by the method. Another advantage is the ability to amplify and detect several genes in the same reaction by performing a multiplex qPCR assay where each target of interest is amplified by a primer and fluorescent probe with distinct label. The simultaneous detection of multiple transcripts reduces the analysis time and decreases the amount of template required. However, multiplex assays require a delicate balance of reagents in the reaction to ensure that all the targeted genes present in the sample are amplified with similar efficiency. In addition, because there is no post-amplification separation step, the multiplex capability of qPCR is limited by the spectral overlap of the fluorescent reporter probes. Another disadvantage of the qPCR method for analyzing gene expression is the inability to obtain absolute expression levels of transcripts. A relative concentration of the target is determined by plotting the observed fluorescence against the cycle number on a logarithmic scale and then comparing to a standard curve. This measured amount of expression must then be normalized to a housekeeping gene from the same reaction to allow comparison of expression between different samples. Because of these limitations, this method is not the optimal choice when absolute levels of expression of numerous targets are needed.

1.3 Microfabricated Systems as Diagnostic Platforms

Despite great advances in the study of gene expression and the development of many methods for the analysis of RNA, the goal of routine analysis of expression for cancer diagnosis is still unrealized. Yet, early detection is very effective against most types of cancers. For example, organ confined prostate cancer is easily treatable, but once the disease progresses to androgen independent metastasis, chances of survival are drastically reduced. In order for expression analysis to live up to its potential in helping reduce the incidence of cancer deaths, methods of expression analysis must become more widely accessible. A

major hurdle remains the high price of these assays, and accordingly reducing costs and analysis time should be the priority in developing expression analysis methods for implementation for diagnostic purposes. Microfabricated devices are especially well suited for this purpose and have great potential in the development of point of care diagnostics.

Since the 1950s, several advancements in lithography-based technologies and microfabrication have been adapted from the semiconductor and integrated circuit industry to progress toward the goal of miniaturized bioanalysis systems [43,44]. These “lab-on-chip” devices strive to replace conventional molecular and analytical methods by integrating several functions on a single device. Microfluidic devices have been used to perform a variety of assays, with applications ranging from biochemical analysis and environmental monitoring to synthetic chemistry [45-47]. This is because microfabrication makes it possible to rapidly build high throughput systems at low fabrication costs. In addition, microfluidic devices enjoy the advantages of reduced reagent consumption and lower sample requirement, which are particularly useful in clinical settings. Small scales also mean short diffusion distances and high surface to volume ratios which enhance reaction rate and sample mixing, generally resulting in faster analysis and response times [48]. Finally, small volumes and microfabrication offer potential for the integration of many functions on a compact system, including sample preparation, amplification, purification, and detection. By performing all processing steps on a single fully integrated system, sample loss and degradation can be minimized, and protocols are simplified. This has great potential for point-of-care diagnostics, which would greatly benefit from systems capable of detecting multiple analytes from complex biological samples.

Compared to conventional methods, integrated microfabricated systems offer several advantages including lower costs, reduced sample and reagent requirements, faster analysis times and increased automation. For these reasons, microfluidic devices will be investigated for the development of a reliable, robust and rapid system of expression analysis that can replace conventional methods and instruments. Methods of fabrication of the device, separation performance, as well as the detection method all impact the overall performance of the integrated microfluidic device for expression analysis.

1.3.1 Fabrication

Substrate properties dictate the design and fabrication of microfabricated devices and thus directly affect their performance. Specific optical properties, biochemical compatibility, and production costs must all be taken into account. Since first demonstrated by Harrison in 1992, the majority of microfluidics CE devices have been made of glass [49]. There are many reasons why glass has continued to be the preferred substrate for fabrication of integrated microfluidic devices. Its high dielectric strength and well-characterized surface chemistry make it easy to perform a variety of assays. In addition, the fact that glass is optically transparent and has low background fluorescence makes it an optimal material for optical detection [50]. While glass fabrication is expensive, the high cost can be amortized in the long run since the final devices are reusable by cleaning. In addition, when manufactured at high volume, glass microchips can be made at a reasonable cost [51].

Recently, disposable materials, especially polymers such as poly(dimethylsiloxane) (PDMS) and poly(methylmethacrylate) (PMMA), have been explored to replace glass because they are widely available and inexpensive [52]. These materials benefit from a

variety of fabrication methods such as soft lithography, hot embossing, and injection molding techniques that are considerably simpler to perform than glass fabrication. However, their application in integrated bioanalytical devices is limited due to their native fluorescent background that restricts detection methods and results in poorer limits of detection. In addition, plastics and elastomers do not easily permit surface modification, making it difficult to perform high-quality separations which often require coating of the capillary channels.

Glass fabrication methods are directly derived from silicon processing steps used in semiconductor applications. The basic underpinning of the process is photolithography, which enables the fabrication of parallelized systems with high precision and in a highly repeatable manner. A typical process for the fabrication of a microfluidic device is shown in Figure 1.1. A glass wafer is first spin-coated with a layer of photoresist. Photolithography is used to define the features on the substrate, such as microfluidic channels for capillary electrophoresis, by exposure to ultraviolet (UV) radiation through a photomask. After removing the exposed area using a developer solvent, isotropic wet etching by hydrofluoric acid (HF) is used to define the channel depth. Reservoirs are created by drilling via holes into the glass wafer, after which it is thermally bonded to a blank glass wafer to create the microfluidic channels and reservoirs.

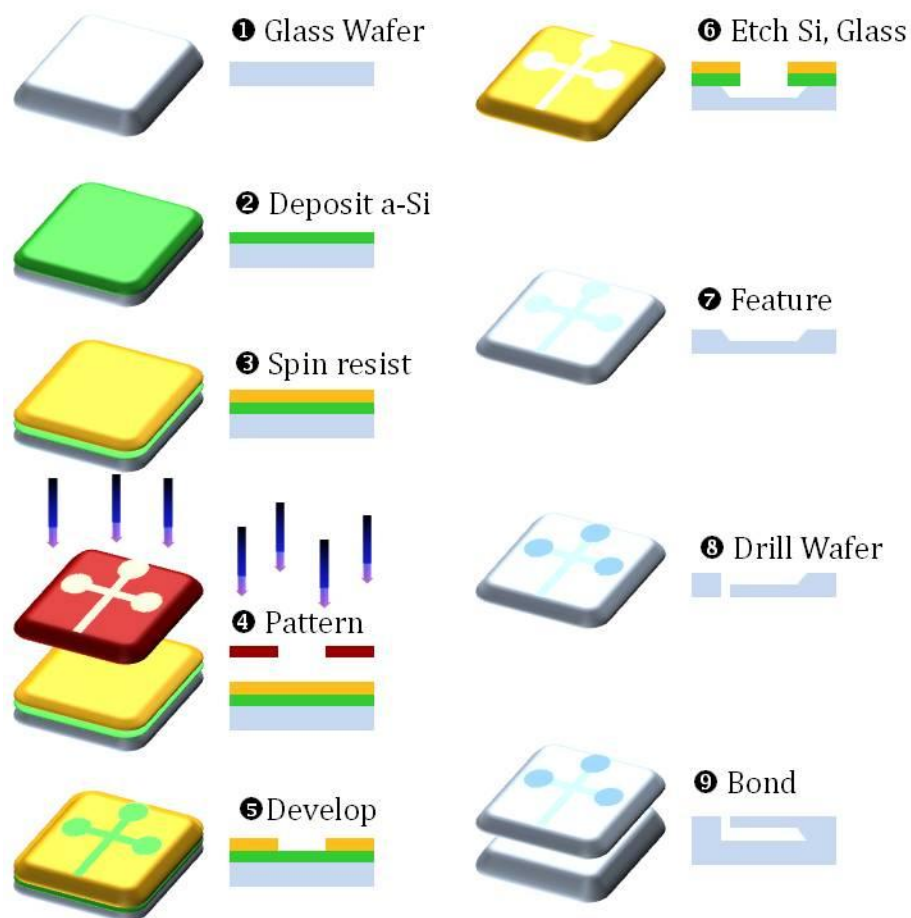


Figure 1.1. Glass microfabrication process. Photoresist is spun on a silicon-coated glass wafer, then patterned by UV lithography. After developing the unexposed photoresist, silicon is removed and the feature is etched into the glass layer. Reservoirs are drilled for fluidic access, and the patterned wafer is then thermally bonded to a blank wafer to create channels.

1.3.2 Capillary Electrophoresis

Cross-linked polyacrylamide gels have been used for size based fractionation of nucleic acids since the early days of DNA sequencing. In fact, the first commercial DNA sequencers were based on a slab gels onto which fluorescently labeled fragments migrated for detection by laser fluorescence excitation [53]. Capillary electrophoresis (CE) increased the throughput of these early technologies. Following Jorgenson and Lukacs' demonstration of the method [54], separation by capillary gel electrophoresis for sequencing was investigated to reduce analysis time, decrease reagent consumption as well as increase automation. Advances in miniaturization technology further revolutionized electrophoretic separations, as microfabricated CE devices provided all of the advantages of conventional CE systems but with the added benefits of micron scale capillary geometry and volumes. For example, a major advantage of microfabricated CE systems is their ability to perform faster separations. This is because analysis time is directly related to the magnitude of the electric field applied to the separation column, and the separation voltage than can be applied is generally limited by the system's ability to dissipate joule heating during electrophoresis. Because microchannels have a high surface-to-volume ratio, they overcome this limitation by being capable of rapid heat dissipation. As a result, CE in microchannels allows the application of higher electric potentials to achieve faster separations and higher resolutions compared to conventional electrophoresis.

Separation performance is measured by the resolving power of the system, which in turn benefits from long separation columns. This effect is most obvious in DNA sequencing, where the quest for high read lengths has led to the development of ever longer capillaries [55]. These however, require longer analysis times and very high potentials which limit their usefulness. In contrast, CE on microdevices boasts high resolution that can be achieved with lower potentials and smaller separation lengths that drastically reduce separation time. In addition, because of the ease of microfabrication, channels can be designed to be folded into compact serpentine structures rather than increasing the size of the substrate to accommodate them [56]. These "hyperturns" enable longer separation channel lengths while minimizing geometric band dispersion, and microfabricated devices using them have been able to demonstrate superior capillary electrophoresis resolution required for performing DNA sequencing.

The proven capabilities of these single channel devices for fast analysis and superior performance have led to a renewed interest in increasing the ability of microfabricated devices to perform parallel analysis. In particular, the goal of low cost genetic screening heightened the interest in developing high-throughput microfabricated capillary arrays for capillary electrophoresis. Microfabrication is ideal for rapidly producing the dense arrays necessary for high throughput analysis. Genotyping array chips capable of analyzing 96 samples in parallel were introduced in 1998 [57], followed by a 96-lane high-resolution sequencing array in 2002 [58]. A larger 384-lane genotyping chip served to exemplify the ability of microfabrication to facilitate parallelism for high throughput systems [59]. Advancements in fabrication technology have also fostered ever-increasing functionality of microfabricated devices. The development of techniques for making miniaturized devices capable of fluidic manipulation and thermal control has resulted in the integration of sample preparation steps on CE devices. The first integrated PCR-CE device in 1996 coupled rapid thermal cycling with electrophoresis to decrease the total analysis time 6-fold [60].

Pneumatically actuated fluidic controls such as microvalves and pumps capable of controlling nanoliter volumes have likewise increased the functionality of CE devices and have accelerated the development of integrated devices for a multitude of applications, including pathogen detection [61], forensic analysis [62], and even space exploration [63].

1.3.3 Detection

Nucleic acids and oligonucleotides can easily be labeled using intercalating fluorescent dyes or by linkage with a fluorescent label. This makes optical detection of biomolecules inside microdevices easily accomplished by direct fluorescence measurements. Advances in fluorescent microscopes and CCD cameras have made this method widely accessible for low cost detection while maintaining flexibility of the microdevice and detection system. However, these methods lack sensitivity and often require large amounts of labeled analytes for detection. Laser induced fluorescence (LIF) addresses this problem by using focused laser light to excite the fluorescent molecules, enabling their detection with high sensitivity. In addition, methods based on LIF allow the detection of several emission wavelengths from a single excitation source. This ability to simultaneously use multiple fluorophores is especially attractive for multiplex assays, as it is another way to discern between different sample components in addition to separation based on CE mobility.

A good example of this type of detection system is the Berkeley rotary confocal scanner built by Dr. Jim Scherer and used in the experiments described in Chapter 2. As shown in Figure 1.2, a 488-nm argon ion laser beam is passed through a dichroic beam splitter and directed up the hollow shaft of a stepper motor. The beam is displaced off the axis of rotation by a rhomb prism and focused on the microchannels by a 60X objective. The collected fluorescence travels back along the same path, passes through the dichroic beam splitter and enters a series of beamsplitters, filters and PMT detectors. This system allows high sensitivity 4-color detection of samples analyzed on microfabricated devices.

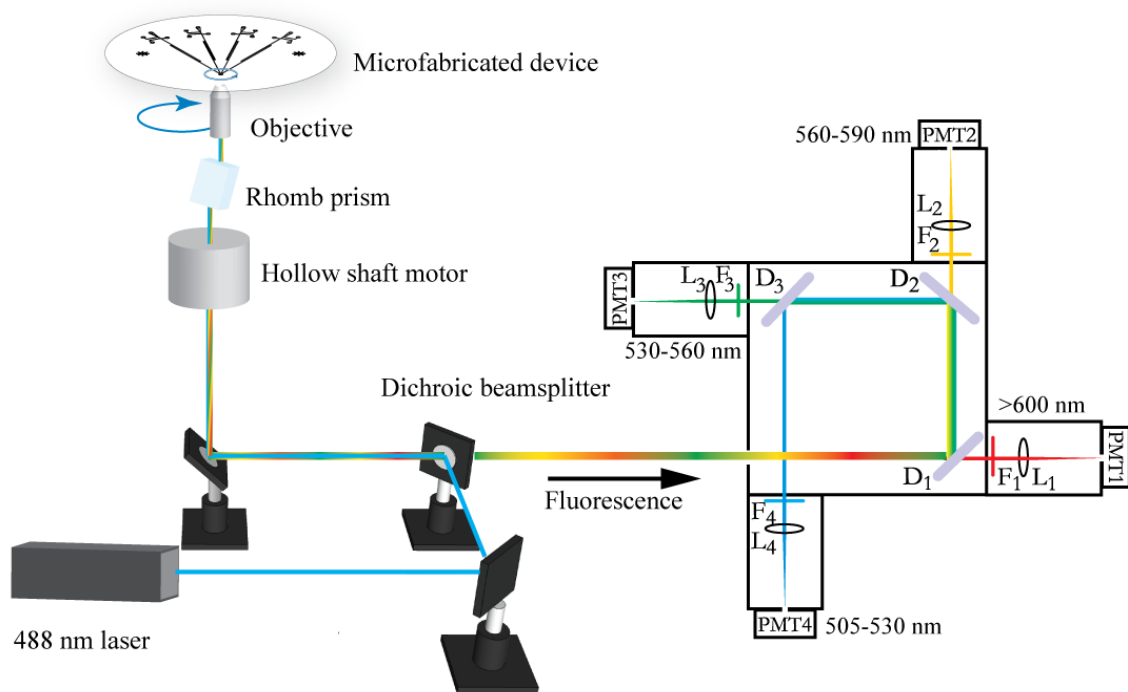


Figure 1.2. The Berkeley rotary confocal fluorescence scanner. Laser excitation is reflected by a dichroic beamsplitter through a hollow shaft stepper motor and deflected by a rhomb prism. Fluorescence is collected by a microscope objective and passes through the same optical path and into the detector assembly. The detector is composed of a series of optics to spectrally and spatially filter fluorescence and 4 PMTs to enable four-color detection. Adapted from [111].

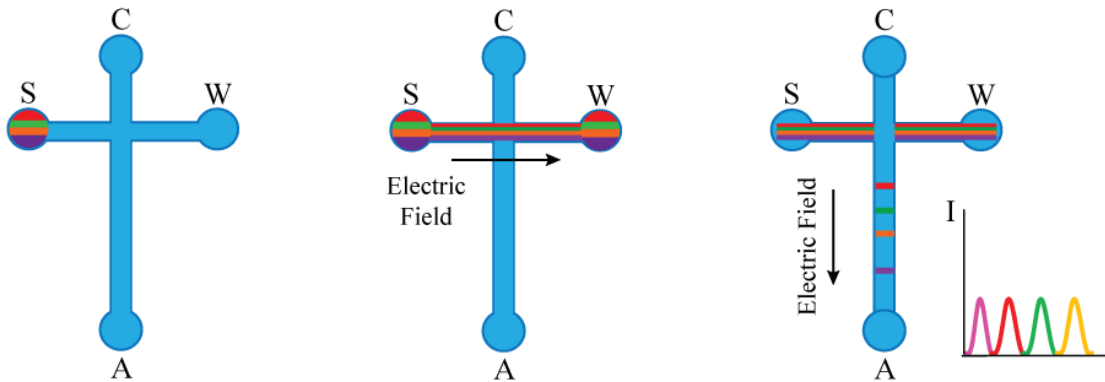
1.4 Integrated Function for High-Performance Detection

A requirement for high performance analysis is effective interfacing of the sample injection with capillary electrophoresis. An efficient injection decreases the sample requirements for analysis and addresses a major drawback of conventional methods which often necessitate large amounts of template. One of the most useful functions that can be integrated on chip is affinity capture, which can increase sensitivity by performing sample concentration and sample clean-up. By improving the sample injection and generating quantitative plugs of purified sample, optimal efficiency of microdevice-based gene expression analysis can be achieved, paving the way for its application in a variety of research and clinical settings.

1.4.1 Sample Injection Methods

The majority of microdevices that perform CE separations use a cross injector to inject sample into the CE channel. This ubiquitous structure permits the formation of small sample plugs for injection of the sample, as shown in Figure 1.3. In the first step, a positive potential is applied to the waste reservoir (W) while the sample reservoir (S) is grounded. This drives negatively charged analytes electrophoretically from the sample to waste, thus filling the microchannel intersection. In the next step, the contents of the pL-sample plug formed in this manner are injected into the separation channel by applying a potential between the cathode reservoir (C) and the anode (A). A small positive potential is also applied at S and W during the run to remove excess sample in the injection arm.

Cross Injection



Affinity Capture

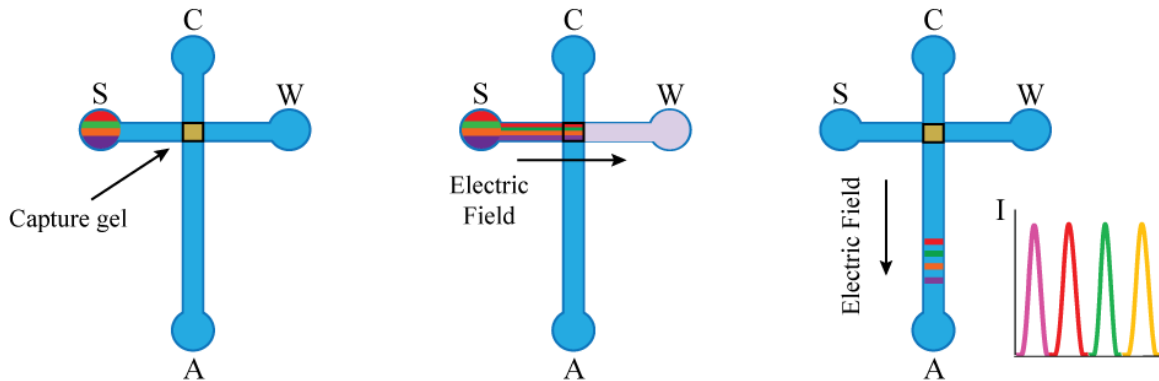


Figure 1.3. Comparison of cross-injection and affinity capture methods. Cross-injection is performed by filling the channel intersection with sample and then applying a separation voltage from the cathode to anode. The sample plug is defined by the channel size and geometry of the intersection. In affinity capture, sample is immobilized on a gel plug prior to electrophoresis. This enables concentration of the sample, resulting in increased sensitivity compared to cross-injection.

One drawback of the cross injection method is that it suffers from electrokinetic injection bias. Small species have high electrophoretic mobility and move into the intersection first. Because these will in most cases be salts and primers, this bias complicates optimization of the injection, and makes it highly sensitive to timing. In addition, cross injection is highly inefficient. Excess sample is necessary to establish enough analyte in the cross injection region. However, the volume injected for analysis is only a small fraction of the total sample, while much of it remains in the reservoirs. As a result, analysis suffers from low sensitivity despite large sample requirements. Moreover, because only a fraction of the total sample is injected and detected, quantification of the results is difficult. Various improvements in injector geometries to achieve better efficiencies and maximize sample use have been investigated [64]. However, the fundamental limitations of sensitivity and quantification of analysis using cross-injection make the method incompatible with the ultimate goal of an integrated point-of-care device.

To address the problems plaguing cross injections, several alternatives have been developed to integrate improved sample injection, cleanup, and preconcentration steps on microdevices. For example, Ueberfeld demonstrated the use of carboxyl-modified magnetic beads for DNA capture and injection for on-chip electrophoresis [65]. Sample purification and concentration have also been accomplished using solid phase extraction columns [66] and membrane filtration [67]. However, the most promising approach for achieving on-chip sample purification is an affinity capture technique that utilizes a functionalized acrylamide gel matrix. This method is based on the integration of a covalently attached affinity probe into the gel matrix during acrylamide polymerization, allowing capture of the desired product in a specific manner. When sample is driven through the capture plug by electrophoresis, it is purified as target molecules become immobilized in the gel matrix while buffers, primers, and non-specific charged products continue to migrate through the gel and into the waste reservoir. The purified and concentrated bound products can then be thermally released for injection into the CE channel and separation.

Two types of affinity capture are made possible in this manner. In the case of oligonucleotide-based capture, the gel is prepared by copolymerizing the polymer solution with a 20-25 nt long capture oligonucleotide probe that is modified at the 5' end with an acrydite element capable of incorporating into acrylamide chains. Because the probe is complementary to a common sequence on the desired products present in the sample, fragments are selectively hybridized to the gel and are immobilized. Multiple fragments can be captured using one probe simply by adding a universal sequence tag on the targets, or alternatively several oligonucleotide probes can be included in the gel for multiplex capture. This method has been demonstrated for sample cleanup of sequencing reaction products, reducing the time necessary for sample purification to only two minutes while generating superior separation performance and read lengths [68]. Alternatively, it is possible to take advantage of the strong affinity between streptavidin and biotin by incorporating streptavidin-modified acrylamide into the capture matrix. As the sample is electrophoresed into the gel plug, all biotinylated products are captured regardless of sequence. This method is especially useful for capturing double stranded products generated by on chip PCR: a biotin labeled strand is captured in the gel while its complementary fluorescently labeled strand can be released thermally for detection. In addition, the universal nature of the capture is an advantage, as it obviates the need for optimization of capture and release temperatures for multiplex assays. Both methods are effective for concentrating the desired

sample components for sample cleanup and increased sensitivity, and when integrated on the microdevice can be a viable alternative to common off-chip cleanup methodologies that utilize magnetic beads and large volumes of reagents.

1.4.2 Photopolymerization in situ

Although gel affinity capture has been pursued to replace traditional cross injection techniques, a challenge continues to be generating the small capture band of sample necessary for high-resolution separations. For example, the use of a side-arm gel capture was an improvement over cross-injection, but the T-shaped affinity injection structure caused band dispersion and distortion during injection into the separation channel [69]. An inline injection system that employs a plug of affinity matrix directly between the cathode and anode in the separation capillary was later shown to be more efficient [70]. However, the device was characterized by low performance as measured by continuous read length. The poor resolution obtained by this technique was attributed to the migration of the negatively-charged capture matrix toward the anode when separation potentials are applied. Moreover, the mechanical properties of the linear acrylamide gels employed caused them to expand at elevated separation temperatures, causing additional band broadening of the injected DNA band. Another disadvantage of these methods was that the capture gel was synthesized off-chip, which necessitated tedious manual gel loading procedures and contributed to low reproducibility.

To exploit the advantages of inline capture, yet eliminate the need for manual loading of capture gel and improve the repeatability and efficiency of the injection, a method for photopolymerization of capture plugs in situ was developed. In conventional polymerization of acrylamide matrices, a chemical agent such as ammonium persulfate decomposes spontaneously in solution, initiating a radical reaction that extends the polymer chains [71]. In contrast, photopolymerization uses otherwise stable photoinitiators that only generate radicals upon irradiation with ultraviolet (UV) light. This allows rapid formation of the matrix while controlling the timing and location of the polymerization.

Photopolymerization has previously been used in microdevices to create sieving matrices for DNA, as well as for protein sizing and hybridization based assays for DNA detection [72-74]. The formation of a capture gel plug by photoinitiated polymerization inside a microdevice is achieved by loading a gel precursor solution containing an acrylamide monomer, crosslinker, initiator and capture probe into the channel by capillary action. The chip is then irradiated with UV light source for several minutes through a chrome mask, allowing the reaction to progress in the unmasked region. After a short (5-10 min) exposure, remaining unpolymerized solution is removed, resulting in the formation of a precisely defined capture gel plug in the unmasked region of the channel (Figure 1.4).

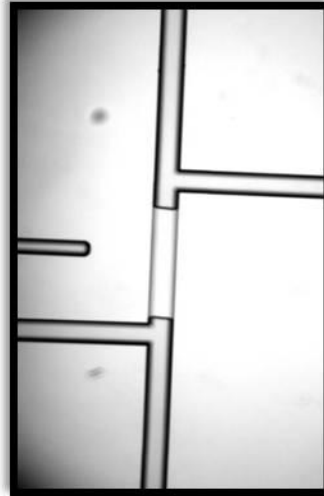


Figure 1.4. A photopolymerized capture gel plug. The photopolymerization method enables the formation of well-defined gel plugs with high control over the location and size of the gel. Here a 500 μm functionalized capture plug for sample concentration by oligonucleotide capture is shown.

A number of reasons make the preparation of the affinity capture matrix by photopolymerization advantageous over off-chip synthesis. First, photolithographic patterning allows precise control over the location, size, and shape of the capture region, and sample plugs can be spatially defined inside the microdevice with high repeatability without having to rely on channel geometry. Second, cross-linked photopolymerized gels are very robust and can easily be anchored to the channel wall by using acrylamide-containing coatings. This increased mechanical strength eliminates gel expansion and electromigration that cause band broadening, thereby improving electrophoretic separation. Finally, because CE performance on microfluidic devices is primarily limited by the separation channel length and by the size of the injected sample plug, the ability to easily produce small gel plugs directly improves resolution of the separation. Microdevices employing photopolymerized capture matrices with CE separation have successfully been demonstrated for the purification and injection of PCR products with near 100% efficiency [75], as well as for increasing sensitivity and resolution for forensic typing [76] and pathogen detection [77].

1.5 Scope of the Dissertation

The goal of the work described in this dissertation is the development of methods for the application of microfabricated capillary electrophoresis devices for cancer detection. Primarily, this work has focused on the detection of tumor-associated biomarkers using gene expression analysis. A microfabricated device is an ideal system for this type of analysis because multiple processes can be integrated on a single device on a small volume scale. Separation by capillary electrophoresis combined with laser-induced fluorescence allows high sensitivity and multiplex detection, and coupled with the integration of a sample concentration and purification step, it is an attractive assay platform for multiplex transcript analysis.

A microdevice incorporating these elements is presented in Chapter 2. A protocol for performing Transcript Analysis with Aid of Affinity Capture (TRAC) is optimized for the purpose of monitoring the expression of genes implicated in cancer. This method is used along with the integrated microdevice to perform multiplex gene expression analysis of several prostate targets. The capabilities of the integrated system are demonstrated by the successful identification of the expression signatures of several established cancer cell lines directly from cells and with high sensitivity.

Finally, the future of integrated microdevices as clinical diagnostic tools for cancer detection is explored. Possible improvements to the system are addressed, as well as methods for expanding the microdevice to a high-throughput microfabricated capillary array. To take full advantage of recent advancements in microfabrication techniques in the areas of low-volume fluid manipulation, on-chip thermal cycling, and high-resolution separation, a fully integrated device is also proposed. By enabling sample preparation, purification, inline injection, and capillary electrophoresis on a single device, total sample analysis time and the number of cells required for analysis are reduced. The fully integrated microdevice could revolutionize the field of point-of-care diagnostics by enabling the rapid and facile detection of comprehensive cancer expression profiles from complex clinical samples.

Chapter 2

Integrated Capillary Electrophoresis Microdevice for
Multiplex Analysis of Cancer Gene Expression

2.1 Summary

A four-channel capillary electrophoresis microdevice that integrates sample cleanup and concentration by on-chip capture coupled with electrophoretic separation is developed for multiplex analysis of gene expression. Prostate cancer genes are assayed by solution hybridization of mRNA with complementary fluorescently-labeled detection probes. Affinity bead capture is used to immobilize and isolate the mRNA-probe complexes. Detection probes are released from the beads and then captured on-chip by an oligonucleotide-modified cross-linked acrylamide capture gel photopolymerized in situ. These cleanup and concentration steps enable efficient injection into the CE channel for separation and four-color detection. Using total RNA extracted from PC-3 cells, prostate gene targets can be detected from as little as 75 ng of starting material. The ability of the assay and microdevice system to evaluate gene expression directly from cell lysates is also demonstrated. The transcript copy number of ten genes was measured in the human prostate cancer cell lines LNCaP, VCaP, PC-3 and 22Rv1, enabling the identification of a distinct gene expression fingerprint for each cell line. The low-volume, high sensitivity, multiplex genetic analysis enabled by this integrated microdevice platform can be extended to cancer diagnostic applications as well as monitoring of disease progression.

2.2 Introduction

Accurate expression analysis of the genes implicated in cancer is important for disease diagnosis as well as prediction of outcome. Identification of biomarkers with expression that correlates with clinical disease facilitates diagnostic tests for early cancer detection. For example, detection of prostate cancer relies heavily on measurements of serum prostate specific antigen (PSA) levels [78,79]. However, this biomarker lacks specificity, and often fails to distinguish prostate cancer from non-malignant conditions such as benign prostate hyperplasia (BPH) [80,81]. Because prostate cancer progresses through distinct stages from pre-invasive disease, to invasive cancer, and then to androgen-dependent or independent metastases [82], the ability to stratify disease by clinical significance is valuable for treatment decisions. For these reasons, a method for multiplex analysis of biomarkers to identify patterns of gene expression is likely to be a more effective approach than single biomarker assays [83-85].

Existing methods for the identification of multiplex gene expression signatures with high sensitivity and specificity all have limitations. Systematic interrogation of expression by microarray analysis [86,87] has been used for large scale biomarker identification [88], profiling of tumor samples [89], as well as detection of cancer gene expression fingerprints [90,91] and prognosis markers [92,93]. However, standard microarray analysis is characterized by low sensitivity [94,95], and relies on reverse transcription to cDNA which can distort transcription profiles [96]. Quantitative PCR (qPCR) features higher sensitivity but likewise necessitates extensive template preparation including transcript amplification which makes quantification of expression less reliable [97,98]. Its use in multiplex assays is further limited by spectral overlap of the fluorescent dyes as well as by diminished robustness of PCR caused by multiple primer pairs [99]. Recently, a method called transcript analysis with aid of affinity capture (TRAC) has been demonstrated for robust and sensitive analysis of a selected set of genes [100]. This method involves solution hybridization with a pool of target-specific probes that are subsequently identified and quantified by capillary electrophoresis (CE). Because this approach features rapid solution hybridization kinetics and does not require RNA purification, reverse transcription, or PCR amplification, it is attractive for the study of gene expression. TRAC has previously been used for transcript analysis of microbial cultures [101], monitoring yeast in fermentation processes [102] and for multiplex quantification of bacterial populations [103]. In those studies, high throughput was achieved by automation of sample processing steps using magnetic bead processors and separation on conventional genetic analyzers. In addition to being cost prohibitive, these commercial devices require large volumes of reagents and sample. Moreover, only a fraction of the prepared sample is injected and analyzed on commercial CE systems, limiting the ability to quantify results. This injection problem also results in decreased sensitivity due to loss of material unless PCR amplifiable probes are used [104]. To address these limitations, as well as reduce costs, sample usage, and analysis time, a microchip-based approach for genetic analysis is desirable.

Advances in microfluidics have enabled rapid, high-throughput, and low cost genetic analysis [50,49]. Analysis on a microdevice enables high performance separation [49,58] as well as the integration of multiple processes, such as concentration for increased sensitivity [105]. On-chip oligonucleotide-based affinity capture prior to electrophoresis [69,70,106] has been used to specifically hybridize detection probes to a complementary probe in a

capture gel photopolymerized in situ. This integrated capture concentration step on a photopolymerized gel has been demonstrated for sample cleanup and injection for forensic short tandem repeat analysis [76,107] and multiplex pathogen detection [77]. These previous studies suggest that a microfluidic analysis device with integrated capture on-chip has potential as a good analytical platform to perform TRAC and to address some of the challenges of the method.

In this study, we develop a microdevice and method for efficient capture, concentration, injection and electrophoresis for sensitive and quantitative detection of selected gene targets. To evaluate the method for gene expression analysis and assess sensitivity and limits of detection, prostate cancer gene targets were detected from total RNA samples. The ability of the assay and microdevice system to analyze cells directly was tested by obtaining gene expression patterns of four established human prostate cancer cell lines. The identification of distinct quantifiable expression fingerprints for metastatic tumor derived cell lines LNCaP, VCaP and PC-3, and organ-confined tumor line 22Rv1 demonstrates the feasibility of using this method to identify gene expression signatures implicated in prostate cancer. This multiplex genetic analysis could also be useful for the direct and quantitative study of other cancers, as well as the characterization of the unique expression signature of many processes, including mechanisms of disease progression.

2.3 Materials and Methods

2.3.1 Microdevice design and fabrication

The microdevice (Figure 2.1A) consists of four analyzers arrayed on a 100-mm diameter glass wafer. The analyzers share a common anode and perform sample injection, concentration and CE separation in parallel on up to four samples. An expanded view of the sample injection and capture region is shown in Figure 2.1B. Each analyzer consists of a tapered 300-nL sample reservoir that is in-line with a 500 μm long double T-junction followed by a separation channel with 10-cm effective length. A 2-mm region between the sample reservoir and the double T-junction facilitates the loading of a backing gel to isolate the capture region from denaturants present in the sample.

The microdevice was fabricated using methods described previously [108]. Briefly, after photolithographic patterning on a 500 μm thick Borofloat glass wafer, features were formed by isotropic etching in 49% hydrofluoric acid (HF) to a depth of 45 μm . Wells were drilled into the fluidic layer, and the wafer was then thermally bonded to a 1.1 mm thick backing wafer.

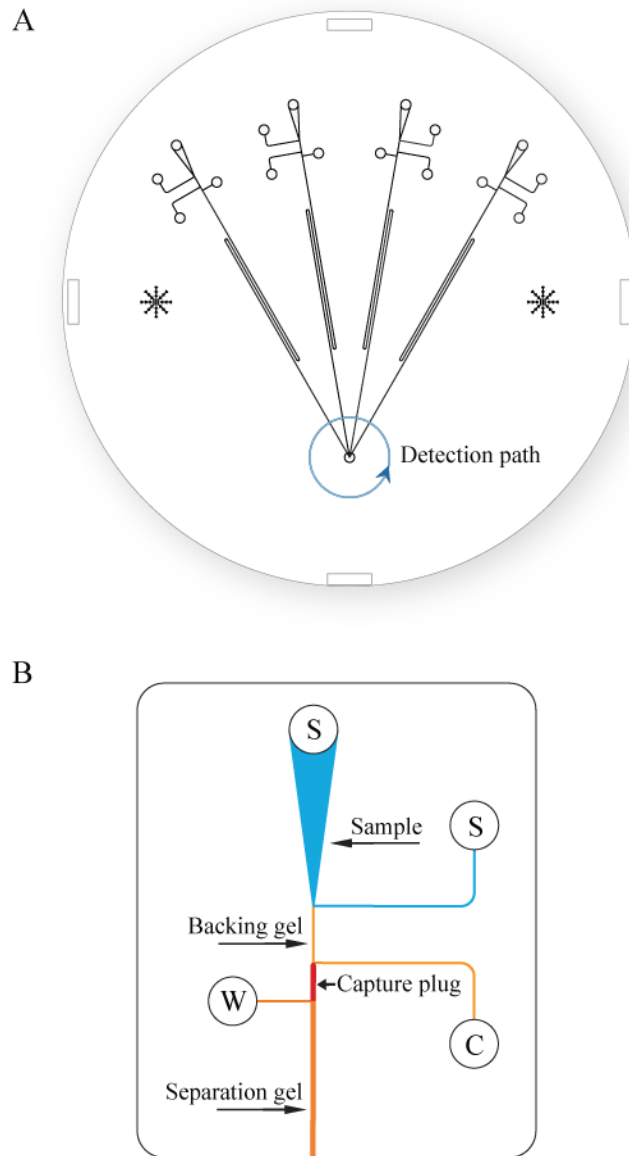


Figure 2.1. (A) Layout of the multichannel capture-capillary electrophoresis (CE) microdevice for gene expression analysis. Four analyzers are arrayed on a 100-mm diameter glass wafer and joined by a common anode for multichannel detection. Each analyzer is composed of a sample reservoir, capture region, and 10-cm long CE channel. (B) Magnified view of the sample injection/capture region. The etched 300 nL sample reservoir is in-line with a 500 μm offset that allows the formation of a capture plug by photopolymerization. A 110 μm wide, 2-mm long channel isolates the capture plug from the sample. Fluidic access points are drilled at the sample (S), cathode (C) and waste (W) wells.

2.3.2 Cell culture and lysis

Prostate cancer cell lines PC-3, VCaP, 22Rv1 and LNCaP clone FGC were obtained from American Type Culture Collection (Rockville, MD) and preserved at the Cell Culture Facility at the University of California at San Francisco (UCSF/CCF). The cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. PC-3 cells were maintained in F-12K Medium (ATCC, Rockville, MD), VCaP cells in Dulbecco's Modified Eagle's Medium (ATCC, Rockville, MD), 22Rv1 and LNCaP cells in RPMI-1640 medium (ATCC, Rockville, MD). All media were supplemented with 10% fetal bovine serum (FBS) and contained 100 units/mL penicillin and 100 mg/mL streptomycin. Confluent monolayers of PC-3 and 22Rv1 cells were washed with 1X phosphate buffered saline (PBS) (ATCC, Rockville, MD), then incubated with Trypsin-EDTA for 5-10 min at 37 °C to dislodge the cells. Loosely attached clusters of VCaP and LNCaP were collected prior to this trypsinization step. Cell pellets were obtained by centrifugation at 2500 rpm for 5 min and washed three times with PBS. After counting cells with a hemocytometer, lysis was performed by adding 1 mL of lysis buffer (Invitrogen, Carlsbad, CA) to 1-2 million cells and disrupting the pellet by shearing. Viscosity of the samples was reduced by passing through a 21 gauge needle using a 1 mL syringe after lysis. Cell lysates were frozen immediately and stored at -80 °C until used in sample preparation.

2.3.3 Probe pool design

Detection probes for cancer gene targets were designed using mathematical algorithms previously published [109]. The final probes were selected using specificity criteria described in earlier work [101,102]; all were 100% complementary to their gene target. Hybridization results were verified by comparison with GeneSapiens, a database of mRNA expression levels based on the results of Affymetrix microarray experiments with total RNA extracted from cell lines [110].

A short FAM labeled probe was also designed to match the in vitro transcribed (IVT) RNA of a rat Gria4 gene serving as an internal standard in the sample preparation. The oligonucleotides probes were synthesized with either a 6-FAM or HEX fluophore label at the 5' end by Biosearch Technologies (Novato, CA). All fluorescently labeled probes included a 20 nt long universal sequence that is complementary to the capture probe contained in the photopolymerized capture gel plug. Final probe lengths were adjusted to optimize separation on-chip and probes were organized into a detection pool for prostate cancer genes as shown in Table 2.1.

Table 2.1: Probe sequences and labels for multiplex detection of prostate cancer genes

Gene	Sequence	Size (nt)	Label
Gria	GTGAACACAGACGGCTCTGCCGATCCCAGCCAACCTTAACCAATT	45	FAM
KRT19	TTATTGGCAGGTCAGGAGAAGAGCCGGGCCAGCCAACCTTAACCAATT	49	FAM
PSA	GAGAAATTGAGTGATATGTCTCCAGGCATGGCCCAGCCAACCTTAACCAATT	52	FAM
GAPDH	AGCACAGGGTACTTTATTGATGGTACATGACAAGGCCAGCCAACCTTAACCAATT	56	FAM
KRT20	ATGTAGGGTTAGGTCATCAAAGACCTTATTCAGGCCTTGCCAGCCAACCTTAACCAATT	59	FAM
SPINK1	CTGATGGGATTTCAAACCTTGTTTCTCAGCAAGGCCCCAGATCCAGCCAACCTTAACCAATT	64	FAM
UPA	CTAGGCTAAAAGGAAGGGATAACTGGCCAAGAAAGGGACATCTAGCCAGCCAACCTTAACCAATT	67	HEX
KLK2	CAAACCTTAGTAAAGCAAGACCATGACATTCACCCACGGAAATCAGAGCCAGCCAACCTTAACCAATT	68	FAM
AR	CTTGAGCAGGATGTGGGATTTTTCTTTTGCATGCAATGATACGATCGACCAGCCAACCTTAACCAATT	69	FAM
STEAP	CACCTTCATGTAACACACCCAGATTTACAATGGCTTAAATGCAAGGAAAAAGCAGCCAGCCAACCTTAACCAATT	73	HEX
ERG	TGTGTTTCTAGCATGCATTAACCGTGGAGAGTTTTGTAAGGCTTTATCAACATCACCAGCCAACCTTAACCAATT	75	FAM

2.3.4 Sample preparation

Variable amounts of PC-3 total RNA (Ambion, Austin, TX) or cell lysates were added to a hybridization reaction with 1 pmol of each fluorescently labeled detection probe and 5x SSC (750 mM sodium chloride, 75 mM sodium citrate), 0.2% SDS, 1x Denhardt (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA, Sigma Aldrich, St. Louis, MO) and 3% Dextran sulphate (Sigma Aldrich, St. Louis, MO) as illustrated in Figure 2.2A. For experiments comparing expression between cell lines, 0.2 fmol of rat Gria4 IVT RNA per reaction was added to provide an internal standard. All solutions were prepared in DEPC-treated water and autoclaved prior to use. Hybridizations were performed in RNase-free tubes at 65 °C for 3 hrs. Following hybridization of the probes with the mRNA in the samples, an affinity capture step was used to remove cell debris, unbound RNA and excess probes. Washed Oligo(dT) coated magnetic beads (Invitrogen, Carlsbad, CA) were added to the sample following the manufacturer's protocol and incubated with continuous rotation at room temperature for 45 min. Beads were collected using a magnet and washed twice with 150 µL of 1x SSC, 0.1% SDS at room temperature, followed by two additional washes with 150 µL of 0.5x SSC, 0.1% SDS. In the final step, washed beads were eluted into deionized formamide (Applied Biosystems, Carlsbad, CA) at 37 °C for 5 min, and the eluate was kept at 4 °C until on-chip analysis.

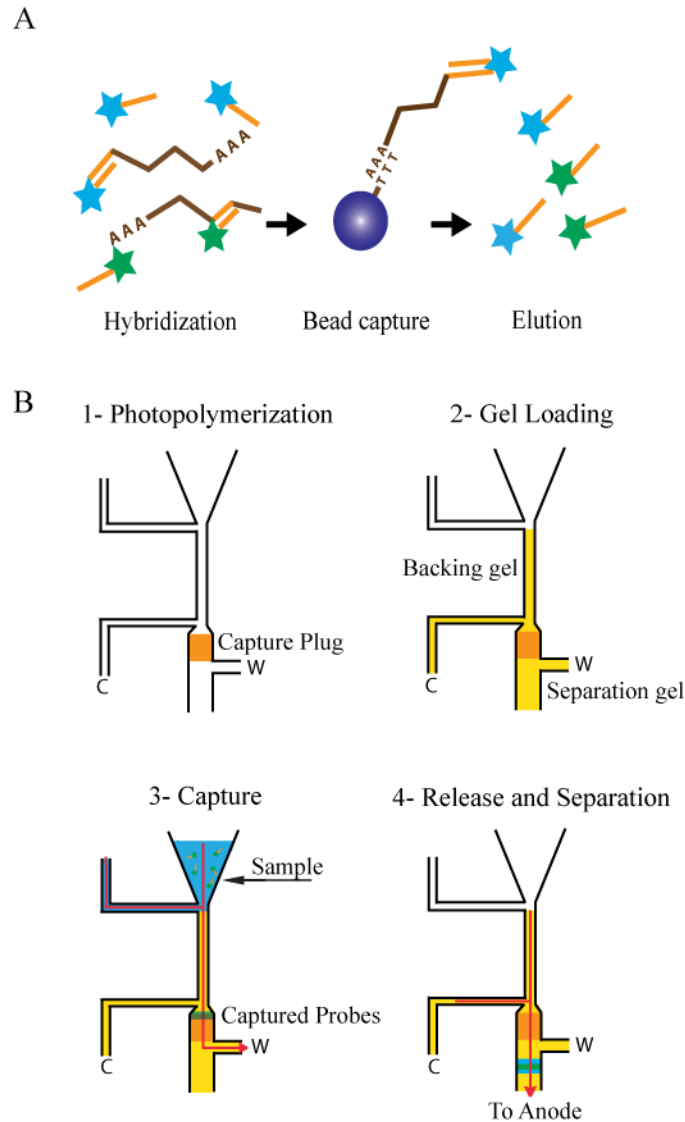


Figure 2.2. Schematic of sample preparation and device operation. (A) Poly(A)-containing mRNAs from cell lysates are first hybridized with a pool of fluorophore labeled detection probes at 65 °C. After affinity capture of hybridized targets by oligo(dT)-coated magnetic beads, unbound material is washed away and the fluorescent probes are eluted from the beads. (B) Microdevice operation: (1) After coating the channel walls, an acrylamide monomer solution is loaded into the channels and the 5%T, 5%C gel plug is formed by photopolymerization. (2) A 6% LPA separation matrix is loaded from the anode and 2.5% LPA backing gel is loaded between the capture plug and sample reservoir. (3) Sample is electrophoresed into the capture plug by applying a field from S to W, and probes containing the universal oligonucleotide sequence are captured. (4) Labeled probes are released at 65 °C, separated at 230 V/cm and detected by laser induced fluorescence on a rotary confocal scanner.

2.3.5 Capture gel preparation

A crosslinked polyacrylamide capture gel was synthesized by in situ photopolymerization as reported previously.[76,75] A monomer solution consisting of 5% (v/v) solution of 19:1 acrylamide/bis-acrylamide (Sigma Aldrich, St. Louis, MO) in 1x TTE (50 mM Tris, 50 mM TAPS acid and 1 mM EDTA) and 500 nmol of a 5' acrydite-modified oligonucleotide capture probe (Integrated DNA Technologies (IDT), Coralville, IA) was prepared in an opaque scintillation vial with Teflon closure (National Scientific, Rockwood, TN). After sparging at room temperature under N₂ gas for 10 min, 0.125% (v/v) TEMED and 0.0006% (w/v) riboflavin (Sigma Aldrich, St. Louis, MO) were added to the monomer solution. To form a 500 μm capture gel plug in the offset double-T junction of each analyzer, the microdevice was aligned under microscope with a chrome photomask (Nanofilm, Westlake Village, CA) patterned with exposure windows. After treatment of the microdevice with a dynamic coating for 4 min (The Gel Company, San Francisco, CA), the monomer solution was introduced into the microchannels and photopolymerization of the exposed region was initiated by irradiating for 10 min with ultraviolet light (10 mW/cm²) from a mercury arc lamp equipped with a 340-380 nm filter. Following exposure, unreacted polymer solution on both sides of the capture plugs was removed from the microdevice and replaced with 1x TTE buffer, completing the photopolymerization step (Figure 2.2B, step 1).

2.3.6 Microdevice operation

After the formation of the capture gel plugs, a separation matrix consisting of 6% linear polyacrylamide (LPA) in 1x TTE was loaded from the anode into the separation channels by applying pressure until the gel filled the waste wells (Figure 2.2B, step 2). A 2.5% LPA in 1x TTE used as a backing gel was loaded manually from the cathode well to fill the microchannel from the top of the capture plug to the bottom of the sample reservoir. A manifold containing platinum electrodes was placed on the chip to electrically connect each of the sample, cathode and waste wells. Prepared sample was loaded into the sample reservoir and wells, and 1x TTE running buffer was placed into the cathode, waste and anode wells prior to electrophoresis. To concentrate the sample by affinity capture, an electric field of 200 V/cm was first applied between the sample and waste wells for 200 s to inject the sample into the top of the capture region (Figure 2.2B, step 3). A smaller electric field of 20 V/cm was simultaneously applied between the cathode and waste wells to prevent loss of the sample to the cathode arm. Affinity capture was performed by lowering the electric field to 100 V/cm to allow the oligonucleotide sequence in the gel plug to hybridize at 30 °C with the complementary universal sequence on the fluorescent detection probes in the sample. These capture conditions were optimized by fluorescence imaging of the sample as it progressed through the loaded microchannel.

After a 12 min capture step, the sample was removed from the sample reservoir and wells and replaced with 1x TTE buffer. The bound products were then washed by electrophoresing buffer through the capture plug for two minutes to remove any unbound material. Following capture, the microdevice was heated to 65 °C to release the fluorescently labeled probes, and a separation field of 230 V/cm field was applied from the sample and cathode wells to the anode. The fluorescent probes were detected by laser-induced fluorescence provided by a four color rotary confocal scanner [111]. The capture

gel plugs and separation matrix were expelled after each run and the microdevice was thoroughly washed with water. The microchannels were flushed with heated piranha solution (3:1 mixture of H_2SO_4 and H_2O_2 , respectively) to restore the glass surface before the next run.

2.3.7 Data processing

Four-color data obtained from the scanner were first processed using BaseFinder v.6.1 [112] to correct fluorescence crosstalk by applying a custom deconvolution matrix. Resulting traces were baseline-corrected and smoothed via a 0.3% Loess filter using PeakFit v4. (Systat Software, Inc., San Jose, CA). Electropherograms were also analyzed with PeakFit to determine the resolution as well as peak efficiency, amplitude and area.

2.4 Results and Discussion

2.4.1 Microdevice and assay design

The microdevice enabled sensitive and quantitative gene expression analysis as a result of integrated on-chip injection, affinity capture and separation by capillary electrophoresis (CE). As shown in Figure 2.2A, samples were prepared by solution hybridization of cell lysates with a pool of target-specific detection probes (Table 2.1). Excess oligonucleotide probes in the reaction provided fast solution binding kinetics, while binding specificity was ensured by the high 65 °C hybridization temperature [103]. Probe-mRNA complexes formed were immobilized on magnetic beads so that excess probes, unbound RNA and cell debris could be washed away. The sample preparation protocol was improved by utilizing the poly(A) tails of intact mRNA to bind the complexes directly to Oligo(dT) coated beads, instead of the streptavidin-coated beads and biotinylated oligo(dT) used in previous work [101,102]. This bead affinity capture step enabled the analysis of total RNA extracts as well as crude cell lysates without mRNA isolation or purification.

Sample concentration by affinity capture was enabled by the use of a photopolymerized gel plug which contains a capture probe complementary to a universal sequence of the detection probes (Figure 2.2B). This target-independent capture eased limitations on the thermodynamic properties of the detection probes and eliminated lengthy optimization of hybridization conditions for the multiplex assay. Because the backing gel isolates the gel plug from the sample reservoir, affinity capture was achieved even with samples that contained denaturants, enabling analysis directly after elution from beads.

2.4.2 Probe pool analysis

Detection of prostate cancer gene targets was accomplished using size based separation and spectral discrimination of their corresponding detection probes. As shown in Figure 2.3, all eleven probes included in the prostate cancer detection pool were resolved with an average resolution of 1.9 between 45 and 75 nucleotides. Detection probes differing in size by a single nucleotide were differentiated with a resolution of 0.9. This high resolution, comparable to that obtained on commercial sequencing platforms [113] is a result of the integration of on-chip affinity capture on a photopolymerized gel plug. Photopolymerization of the polyacrylamide capture gel allows precise control of the dimensions of the 500- μ m capture plug [72,73]. The captured detection probes are thus confined to a small injection band that is in-line with the separation channel, minimizing injection band broadening and improving separation [76]. The robustness of the gel plug also eliminated the problem of gel expansion that has been shown to reduce resolution in previous work [114].

Multiplex detection was achieved by combining CE separation of probes with multicolor detection of both FAM (blue) and HEX (green) fluorescent labels. Separation of the probe pool was characterized by average peak efficiency of 2×10^4 theoretical plates/cm. Peak capacity, defined as the maximum number of analytes that can be detected by the system, was determined to be 45 in the 310-380 s time interval. Additionally, the number of gene targets analyzed could be doubled by detecting two additional fluorescent labels on the

four-color rotary scanner. This theoretical calculation suggests that the microdevice system is well suited for multiplex analysis.

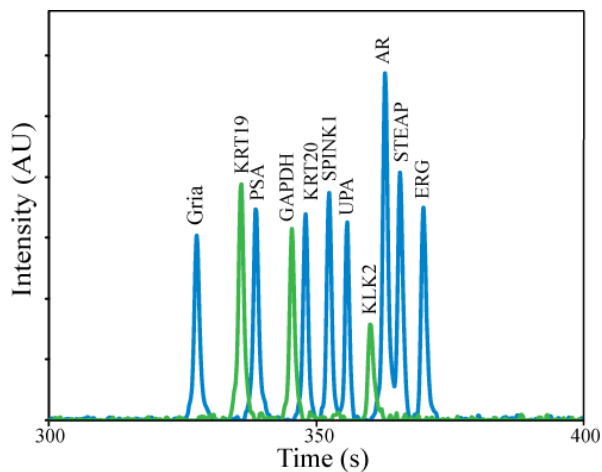


Figure 2.3. On-chip separation of the prostate cancer probe pool. Electropherogram shows that all eleven detection probes are resolved by electrophoresis at 230 V/cm on a 6% LPA gel with an average resolution of 0.9. Multiplex analysis is achieved by detecting two spectrally resolved fluorescent labels, FAM (blue) and HEX (green).

2.4.3 Analysis of total RNA samples

The successful detection of gene targets from total RNA samples is demonstrated in Figure 2.4. Total RNA extracted from PC-3 cells was hybridized with the full prostate cancer detection probe pool then analyzed on-chip directly following elution. Peaks generated by CE separation were identified by comparing with the migration times of the detection probes separated on-chip under the same electrophoretic conditions. As shown in Figure 2.4A, three large peaks were identified, indicating the high expression of cytokeratin-19 (KRT19) and urokinase-type plasminogen activator (UPA) genes in addition to the glyceraldehydes 3-phosphate dehydrogenase (GAPDH) housekeeping gene. Two small additional blue peaks were also detected from 2 μg of total RNA but were not detectable at lower amounts of sample, indicating that the genes STEAP and ERG are expressed in the cells at low levels. Other prostate cancer genes targeted by the probe pool were not detected. The pattern of gene expression in PC-3 cells uncovered by this analysis was confirmed by comparing to GeneSapiens, a database of mRNA expression levels determined by Affymetrix microarray experiments [110]. While this database does not show quantitative expression levels, relative abundance of KRT19, UPA and STEAP were consistent with results observed.

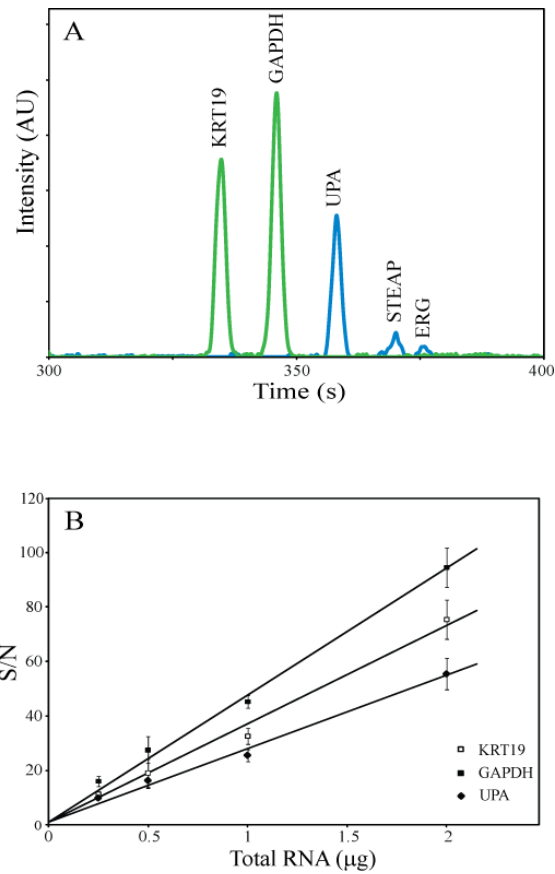


Figure 2.4. On-chip analysis of total RNA samples from PC-3 cells. (A) Electropherogram shows the gene expression pattern for the PC-3 cell line obtained with 2 μg of total RNA. As expected for this cell line, the cells show high expression of genes KRT19 and UPA in addition to the housekeeping GAPDH gene. Low amounts of STEAP and ERG are also detectable, while other prostate cancer genes targeted by the probe pool are not detected. (B) Assay sensitivity determination. Samples are prepared with 250, 500, 1000, and 2000 ng of total RNA ($n = 3$ for each data point), and the S/N ratios for each of the detected probes at the starting sample amounts are shown. This analysis shows that the microdevice can be used to detect the expression pattern of PC-3 cells without amplification with only 250 ng of totRNA sample, with $S/N > 10$.

2.4.4 Assay sensitivity

Assay sensitivity was determined by varying the starting amount of total RNA used in the hybridization reaction and measuring resulting signal to noise ratios. Figure 2.4B shows the S/N for the KRT19, GAPDH and UPA probe peaks detected in the total RNA of PC-3 cells over the range of 250 ng to 2 μ g of starting total RNA amount. The results show a linear relationship between the amount of input sample total RNA and fluorescence intensity of the probe peaks. In addition, the pattern of gene expression obtained from the total RNA of PC-3 cells was maintained over the range of concentrations studied. All three peaks included in the expression pattern for PC-3 were detected on the microdevice with S/N > 10 with only 250 ng of starting total RNA sample, demonstrating the high sensitivity of the system. Extrapolation of the obtained linear regression for S/N = 3 yields calculated limits of detection for the KRT19, GAPDH and UPA genes of 55 ng, 45 ng, and 75 ng, respectively, meaning that as little as 75 ng of total RNA sample can be used to identify the gene expression pattern for the PC-3 cell line. This limit of detection is lower than the 1-10 μ g typically required for standard microarray analysis. Although not as sensitive as real-time PCR, these results were obtained without transcript amplification. The high sensitivity of the on-chip analysis stems from the integrated photopolymerized capture plug which serves to concentrate the sample as it is injected by electrophoresis into the device, resulting in a 3-5 fold increase in signal intensity [75]. These results demonstrate the suitability of the assay and microdevice system for sensitive analysis of gene expression without amplification.

2.4.5 Detection of gene expression fingerprints directly from cells

To demonstrate the ability of the assay and microdevice system to perform gene expression analysis of cells, samples were prepared from four prostate cancer cell lines then analyzed on-chip. A key advantage of this approach is the ability to generate samples ready for on-chip analysis directly from crude cell lysates without having to change sample preparation protocols. Cell lysates were hybridized with the detection probes in the same manner as the total RNA samples; the only modification was an increase in time allowed for the magnetic capture of bead-RNA complexes before the wash steps to compensate for the increased sample viscosity. The electropherograms in Figure 2.5 demonstrate successful detection of prostate cancer genes expressed in the cell lines LNCaP, VCap, 22Rv1 and PC-3, highlighting the ability of the microdevice to perform sensitive gene expression analysis from cells without amplification. In order to detect genes expressed at low levels, the expression patterns shown were obtained with 30,000-40,000 cells; however, cell lines were generally identifiable with less than 20,000 cells in the sample. In addition, the direct use of cell lysates in the assay allowed rapid and simple sample preparation by obviating the need for lengthy RNA purification procedures.

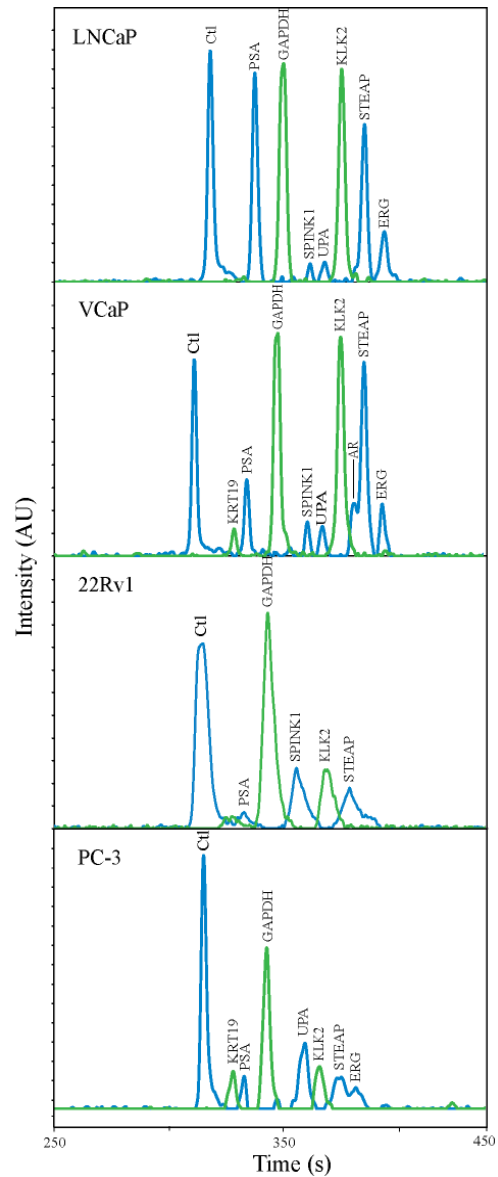


Figure 2.5. On-chip analysis of cell lysate samples showing expression patterns for the prostate cancer cell lines LNCaP, VCaP, 22Rv1 and PC-3. Samples are prepared with 30,000 - 40,000 cells. Genes expressed in the cells are identified by the fluorescent label and mobility of their corresponding detection probes. Control (Ctl) peak from an in-vitro transcribed (IVT) RNA serves as an internal standard. Patterns obtained match expected expression of target genes and reflect well-established differences in androgen sensitivity among the cell lines.

Prostate cancer genes expressed in the cell lines were identified on the CE trace based on migration time. The addition of an in-vitro transcribed (IVT) RNA of the rat glutamate receptor (Gria4) gene, used as an internal standard, enabled the quantitative analysis of expression of individual transcripts in the cell lines. IVT RNA is a better internal control than GAPDH, as the expression levels of GAPDH are highly variable, even among cell lines [115,116]. Peaks for the detected probes were first normalized to the standard peak then quantified based on the number of molecules of IVT RNA present in the sample hybridization. Because peak shape and width showed variation between runs, peak areas were used for accurate quantification of probes. Assuming perfect hybridization and capture efficiency, an absolute measure of expression of each transcript in copy number per cell can be obtained. The resulting gene expression levels for the prostate cancer cell lines LNCaP, VCaP, 22Rv1 and PC-3 are quantitatively compared in Figure 2.6. A comparison of absolute expression between different genes would also be possible after further quantification of the sequence-dependent hybridization efficiencies of the probes used in the TRAC assay.

This analysis allowed the identification of repeatable and recognizable expression fingerprints of the prostate cancer cell lines that matched expression information available on GeneSapiens. While that database and others corroborate the relative expression of the selected genes, this study is the first to quantify them in copy number/cell across these four established cell lines. As shown in Figure 2.6, the results demonstrate differences in the expression of the androgen receptor (AR), which reflect the well-established differences in androgen sensitivity and dependence among the cell lines analyzed. The cell line PC-3, a model for androgen insensitive tumors, does not express the androgen receptor, while AR insensitive 22Rv1 expresses it in low copy numbers [117]. VCaP cells, also originated from a hormone-refractory tumor [118] express AR [119], while LNCaP cells, which have been shown to be androgen responsive but not androgen dependent [120] express AR in very small quantities. Expression of the androgen receptor is significant in the study of prostate cancer, as it plays a well-established role in the prostate epithelium and is a key therapeutic target in cases of advanced and metastatic disease [121].

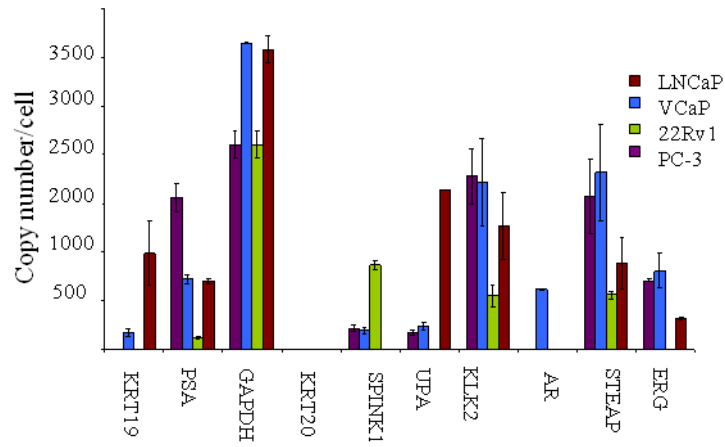


Figure 2.6: Expression patterns for prostate cancer cell lines determined by on-chip analysis. The number of copies of prostate cancer genes is quantified by normalizing to an in-vitro transcribed (IVT) RNA of known concentration. The androgen- responsive LNCaP and VCaP cell lines express high levels of PSA and KLK2, as well as the prostate specific genes ERG and STEAP. 22Rv1 cells express low copy numbers of those genes but high levels of the SPINK1 gene. PC-3 cells are characterized by high copy numbers of UPA and KRT19.

Androgens also affect the expression of other genes, as they regulate prostatic epithelia cell function, including the secretion of prostate specific proteins such as prostate specific antigen (PSA) [122]. PSA expression was observed to be highest in cell lines VCaP and LNCaP and low in 22Rv1 [123]. VCaP and LNCaP also showed high expression of six-transmembrane epithelial antigen of the prostate (STEAP), a hormone-independent prostate specific gene highly expressed in advanced prostate cancer, that in previous studies had been detected in LNCaP with a high copy number and in PC-3 at lower levels of RNA expression [124]. In addition, the VCaP cell line was characterized by the significant expression of the transcription factor ERG, which is overexpressed in clinical prostate cancer [125] and has recently been implicated in a gene fusion observed in subset of androgen-dependent prostate cancer [126]. In contrast, the cell line 22Rv1 showed lower copy number of PSA and kallikrein-2 (KLK2), both AR-regulated genes, than VCaP and LNCaP, as expected [123]. 22Rv1 cells were also distinguishable by the presence of the serine protease inhibitor (SPINK1) gene, whose increased expression has been determined to be a significant predictor of prostate cancer [127], and which has been used as a biomarker for urine-based diagnostic tests [128]. Although it has been proposed as a marker for discerning prostate from urothelial carcinomas [129], expression of cytokeratin-20 (KRT20) was not observed in any of the cell lines studied.

The PC-3 cell line is known for its reduced expression of common prostate cancer biomarkers such as AR and PSA. As already indicated by the total RNA analysis, cytokeratin-19 (KRT19), a gene whose involvement in neoplastic progression of human prostate epithelial cells is well-established [130], and urokinase-type plasminogen activator (UPA), a serine protease secreted by prostatic epithelium that plays a role in tumor invasiveness of hormone-dependent malignancies [131], are the targets expressed in highest copy numbers in these cells. The observed high expression of UPA also supports the previous finding that PC-3 cells secrete high levels of UPA while prostate cells with lower metastatic potential such as LNCaP do not [132]. However, the analysis of PC-3 cells also revealed expression of PSA and KLK2 which was not observed in the total RNA analysis of the same cell line and in copy numbers higher than expected for this androgen-independent cell line. This difference is explained by the often-noted biological variation among batches of cells of the same line. In particular, it has been established that androgen-sensitive expression of prostate epithelial cells is strongly affected by cell culture conditions such as medium and cell density [133]. Nevertheless, these results demonstrate the ability of the assay and microdevice system to generate consistent gene expression fingerprints from cell samples with high sensitivity, opening the door to other applications such as the identification of tumor cell expression signatures that can be used to predict clinical outcome of prostate cancer [89].

2.5 Conclusions

We have demonstrated a robust assay and integrated microdevice for multiplex gene expression analysis. The photopolymerized oligonucleotide-modified capture gel plug enables the concentration of multiple detection probes for high sensitivity and efficient injection. The method enables analysis of both RNA and cell lysate samples without RNA purification, reverse transcription or PCR amplification. The excellent separation efficiency indicates the microdevice is well-suited for performing high order multiplex analysis. This microdevice and assay system is a versatile platform for highly sensitive and quantitative transcript analysis and can be extended to other applications such as testing tumor samples and monitoring expression fingerprints for diagnostic purposes.

2.6 Acknowledgements

Microfabrication was performed at the University of California Berkeley Microfabrication Laboratory. We thank Dr. Kristiina Iljin for the detection probe sequences and Dr. Juha Kotimaa for providing the IVT RNA. We also thank Dr. Hans Soderlund, Dr. Harri Siitari, Dr. Lotta Amundson, Dr. Jari Rautio and Dr. David Duberow for valuable discussions. Funding for this work was provided by the Technical Research Centre of Finland (VTT).

Chapter 3

Prospects

3.1 Introduction

The previous chapters have established the feasibility of performing gene expression analysis on an integrated microfluidic platform to achieve rapid and multiplexed mRNA detection with high sensitivity. The applicability of the method and microdevice system to the quantitative detection of cancer biomarkers was further validated by the identification of cancer expression signatures. In this final chapter, I discuss the prospects for future applications of this system in the clinical setting. Major concerns of point-of-care diagnostic systems include reducing sample requirements as well as decreasing costs and analysis time. To address these issues, I propose integrated devices capable of PCR amplification and on-chip nanoliter scale sample processing, coupled with quantitative sample injection and separation by CE. In addition, these microfluidic bioprocessors are scalable to multichannel microdevices for high-throughput analysis. By taking full advantage of recent advancements in microfabrication techniques in the areas of on-chip thermal cycling, low-volume fluid manipulation and capture gel photopolymerization, these integrated microsystems will eliminate the inefficient sample manipulation currently required for analysis and enable the rapid and facile detection of comprehensive cancer expression profiles from complex clinical samples.

3.2 Increasing Sensitivity for Analysis of Clinical Samples

Most gene expression methods rely on sample amplification by PCR for sensitive detection. For example, analysis by microarray often includes one or two amplification steps to reduce the amount of sample required. However, as discussed in Chapter 1, amplification of the sample has several drawbacks, including biasing of the transcription profile. The results presented in Chapter 2 demonstrated that sensitive detection of expression patterns can be accomplished without PCR amplification by using the TRAC method in conjunction with the integrated capture-CE microdevice. The lack of amplification was a valuable advantage as it enabled the absolute quantification of transcript levels. However, the analysis of most clinical samples requires even higher sensitivity detection. In particular, in order for the microdevice to be useful as a diagnostic test, it must be able to generate results from a small number of cells to minimize the invasiveness of the sample collection procedure. For example, non-invasive detection of prostate cancer is possible by analyzing prostate cells that are shed into urine [128]. However, while these urine-based diagnostic assays are preferable over painful biopsies, their adoption is limited by the challenge of obtaining an accurate measure of expression from the few cells present in the sample. Because of the heterogeneous nature of tumors, the detection of metastatic cells would likewise be facilitated by more sensitive methods of gene expression analysis.

To increase the sensitivity of expression analysis by TRAC while maintaining the ability to quantify gene transcripts, the isolated detection probes obtained by TRAC hybridization can be used as the template for PCR instead of amplifying the original sample. Using this approach, hybridization with the detection probes is still performed on cells directly, followed by bead immobilization of mRNA-probe complexes. However, after elution from the beads, the probes are amplified by PCR rather than analyzed directly. A small number of amplification cycles is performed in order to maintain linear response. Because the released detection probes accurately reflect the transcript levels present in the initial sample, using them as the template for the amplification reaction yields an accurate interpretation of the expression profile. Kataja et al. previously demonstrated the feasibility of this approach by performing 16 cycles of PCR amplification on TRAC hybridized and eluted probes prior to injection on a commercial genetic analyzer [104]. Results of those experiments showed a 20-fold increase in the sensitivity of the assay while maintaining the stoichiometric ratios between the probes. The method further benefits from the fact that the detection probes already include a universal sequence for affinity capture. This permits the use of a common pair of primers to perform robust multiplex PCR, resulting in highly reproducible amplification [134]. With proper optimization of the PCR protocol, the amplification of the cancer detection probes will therefore enable sensitive and quantitative detection that is a superior alternative to RT-PCR of the target RNA sample. Kataja et al. reported limits of detection of 0.5 ng of mRNA or roughly 500 cells [104]. By using this method on a microfluidic device capable of efficient injection, the cellular limits can be further reduced. For example, using PCR amplification integrated with affinity capture, Toriello et al. demonstrated gene expression measurements at the single cell level [135].

Because PCR amplification is extremely temperature sensitive, high-precision control of the reaction temperature during thermal cycling is necessary. In particular, deviation from the melting temperature of the primers or the optimum temperature of the DNA polymerase strongly affects PCR performance. As a result, integrated microfabricated

devices capable of PCR amplification must include necessary components for heating and cooling, as well as temperature sensing. These functions can be provided by instrumentation external to the microdevice itself. For example, the use of infrared (IR) radiation as a heating mechanism for performing PCR has been demonstrated by Easley et al. [136], and IR thermal cycling systems have been used for infectious disease detection [137]. However, because this technique requires an external source of focused radiation, the applicability of such microdevices outside the research setting is limited. In contrast, contact heating methods have been extensively pursued for portable microsystems. In particular, resistive thin film heaters benefit from a variety of fabrication methods and are well-suited for integration on microdevices. Thin-film heaters made of Ti/Pt [138], polysilicon [139], or transparent Indium Tin Oxide (ITO) [140] can be microfabricated to accommodate a variety of design constraints and applications. Resistive temperature detectors (RTD) can likewise be patterned directly onto the device for temperature monitoring, enabling the accurate temperature control required for high thermocycling performance.

The integration of the heating elements necessary for thermal cycling on the same device as the CE separation channel makes a compact analysis system possible. An example of a microfabricated PCR thermocycler is shown in Figure 3.1. Microfabrication of integrated heaters and RTDs on the surface of a monolithic glass substrate is straightforward and has been reported previously [138,141]. PCR heaters are made by patterning Ti/Pt elements on the backside of a wafer containing an etched PCR chamber. Gold electroplating is used to minimize heating of the heater contact pad and to ensure localized and uniform heating over the PCR reactor. Photolithographically patterned four-point RTDs are placed parallel to the PCR chamber to allow accurate monitoring of the temperature inside the reactor and to guide precise thermal cycling. By integrating heaters and RTDs on the same device, the overall thermal transfer from the heating elements to the PCR chamber is improved. For instance, the first PCR microdevice by Lagally et al. reported increased heating rates of 20 °C/s [141]. In addition to reducing the amount of time necessary for completing the amplification cycle, this fast thermal response time facilitates highly efficient and balanced amplification of the sample.

The first integrated sequencing device by Blazej et al. showcased the complete integration of PCR amplification, product purification, and separation [142]. By performing thermal cycling for multiplexed Sanger extension in a 200 nL reactor, the microdevice was able to produce over 500 base reads from only 1 femtomole of DNA template. More recent work improved the injection efficiency of the thermally cycled products by utilizing the in-line affinity capture method, thus enabling sequencing from only 100 attomoles of template [70]. These results are promising for the similar microdevice proposed here: first, the eluted TRAC detection probes are loaded into the PCR chamber along with PCR reagents for thermal cycling. After amplification, the extension products are pumped into a hold chamber. An electric field is then applied to drive the amplified probes through a photopolymerized gel plug functionalized with an oligonucleotide capture probe. As the sample is electrophoresed through the capture matrix, the detection probes are immobilized using a common capture sequence while primers, buffer and unincorporated dNTPs from the PCR reaction are washed away. By combining the amplification of purified TRAC probes with a highly efficient and quantitative injection into the separation channel, this microdevice will enable the identification and quantification of gene expression levels with

high sensitivity. In addition, the rapid low-volume thermal cycling and integrated electrophoretic separation will enable fast analysis of samples. Based on the results of previously published integrated PCR-CE microdevices, the proposed microdevice is expected to complete expression analysis in 1.5 h with a detection limit of as low as 20 copies [143]. This is superior to current methods of transcriptional analysis and would facilitate the detection of expression fingerprints from clinical samples containing only a few cells, expanding the applicability of the microdevice to a wider range of clinical samples and enhancing its potential as a point-of-care diagnostic assay for cancer detection.

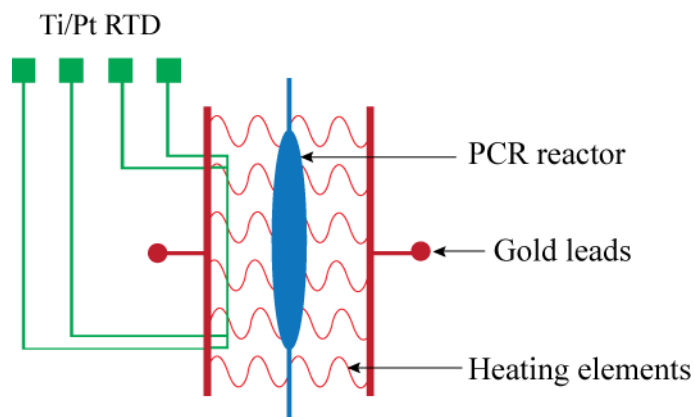


Figure 3.1. A microfabricated PCR thermocycler. A heater with Ti/Pt heating elements and gold contact pad is patterned on the backside of a glass wafer for uniform heating of an etched PCR reactor. A four-point Ti/Pt patterned RTD enables accurate monitoring of the temperature inside the PCR chamber. The integration of microfabricated heating elements on the same device enables rapid thermal cycling for efficient sample amplification.

3.3 Fully Integrated Cancer Detection for Point-of-Care Testing

While the PCR-capture-CE microdevice proposed above will be capable of highly sensitive and multiplexed gene expression analysis benefiting a variety of applications, significant off-chip sample preparation remains necessary prior to analysis on-chip. The final step in achieving the goal of fully integrated analysis is to supplement the microdevice with the capability to perform all the necessary sample preparation steps. Gene expression analysis by the TRAC method offers the advantage that cell lysates can be used directly. The straightforward sample preparation protocol simply requires solution hybridization of components, followed by bead affinity capture. Microfabricated components for mixing, pumping, and microparticle manipulations developed in the Mathies group are utilized to integrate these functions with on-chip separation and analysis. By including these processing steps at the front-end of the microdevice, the macro-micro interface problem is eliminated and the efficiency of the analysis is enhanced. The fully integrated device proposed here will be able to perform complete analysis of gene expression from a raw cellular sample for a truly sample in-answer out point-of-care diagnostic.

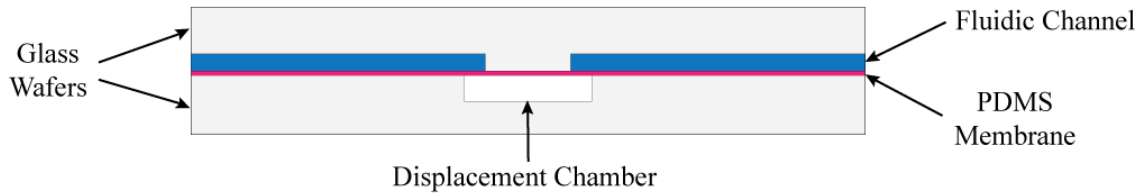
One of the challenges facing the proposed microdevice is the seamless integration of several analytical steps. This includes physically separating each functional unit of the fully integrated system while maintaining the ability to transport reagents across components and minimizing sample loss and dilution [50]. The integration of microfabricated valves plays a key role in accomplishing this goal. Microvalves can be categorized by their mechanism of operation: active mechanical valves include electromagnetic [144], piezoelectric [145], and pneumatic [146,147] varieties, while passive mechanical valves typically consist of check valves [148]. Non-mechanical valves can be active, usually by utilizing a phase change material [149], or passive such as hydrophobic [150] and gel valves [151]. Selecting the appropriate microvalve must take into consideration ease of fabrication, as well as the physical properties of the valve such as pressure resistance and dead volume which dictate the volumes that can be handled by the device. Two types of microvalves will be used in the proposed fully integrated microdevice. First, a passive valve composed of a viscous gel matrix between the PCR chamber and the separation channel will be used to prevent contamination of the PCR reactor by coating and photopolymerization reagents, as well as to avoid leaking from the reactor during thermal cycling [77]. In addition, the new microdevice will include pneumatic microvalves to efficiently move solutions between different components. Such microvalves have been extensively employed in integrated microsystems, where their superior abilities in manipulating nanoliter-quantities of solution are well-established [152]. Their integration on this microdevice will enhance the transport efficiency of the sample and reagents during sample processing and analysis.

The design of the microvalves used is critical for their large-scale integration into the glass microsystem as well as for easing limitations on sample processing. In particular, the microvalves selected must be suitable for the analysis of biological molecules, and consequently, the materials used in fabrication must prevent diffusion and adsorption that would lead to contamination of the microdevice components. The monolithic elastomer membrane valves developed in the Mathies group address this concern [153]. These microvalves are formed by bonding a featureless polymer membrane between two patterned glass wafers; one of the glass wafers features a discontinuous channel structure while a displacement chamber is etched into the other. As shown in Figure 3.2, each valve consists

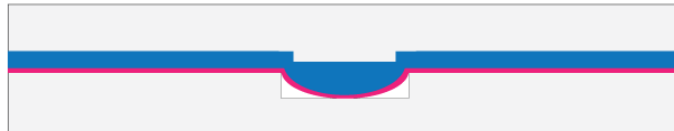
of a displacement chamber which extends over a gap in the channel. When the valve is at rest, a thin PDMS membrane between the glass layers isolates the chamber from the fluidic channel and the flow is interrupted. The valve is operated pneumatically: when a vacuum is applied to the displacement chamber, the membrane deflects away from the discontinuity, allowing the valve to fill and permitting fluid flow. These robust valves seal reliably even against high fluid pressures [154], making them ideal for restricting sample flow and compartmentalizing reagents on the integrated microdevice.

The functionality of the microvalves can further be expanded by arranging three valves in series to form a self-priming pump. On the integrated microdevice, these micropumps are used to pump sample, deliver reagents, and transport solutions between various compartments of the microchip in an automated fashion. The volume of solution pumped is a function of the valve dimensions and the flow rate, and can be adjusted by tuning the actuation time [153]. In previous studies, this micropump approach has enabled pumping of volumes ranging from nanoliters to a few microliters, and has demonstrated rates of up to 100 nL/s. Because the contents of a single microvalve can be transferred in a discrete manner through a series of microvalves, automated sample processing is also made possible [155]. This use of microvalves has already benefited several applications, including integrated DNA sequencing [142], pathogen detection [156], and single cell genetic analysis [157], and will similarly enable sophisticated washing and dilutions protocols necessary for performing TRAC sample preparation on the fully integrated microdevice.

Atmospheric Pressure



Vacuum Applied



Micropump

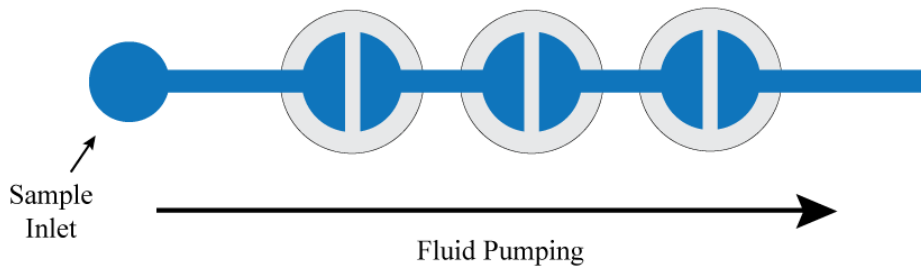


Figure 3.2. Microfabricated valves for fluid isolation and pumping. Each valve consists of a discontinuity in the fluidic channel which is bridged by a displacement chamber. A thin PDMS membrane interrupts the fluidic flow when the valve is at rest. The valve is activated by applying a vacuum to deflect the membrane and allow flow in the channel. A microfluidic pump is generated by activating three valves in series and can be used to move a discrete volume of sample.

Due to the laminar flow profiles characteristic of microfluidic systems, active control of mixing will be necessary for combining reagents on-chip. In particular, a microvalve strategy for mixing will also be employed for the hybridization of magnetic beads with the sample. Pumping fluids through a circular channel path is a common method for active mixing [158], and mixing loop structures have previously been used for performing serial operations [159]. Grover et al. illustrated the synergistic potential of microvalves for sample clean-up by trapping functionalized magnetic beads in a microfluidic processor to capture complementary oligonucleotides from a recirculating concentrated DNA solution [160]. One of the advantages of this method is that it allows large sample volumes to be manipulated on-chip using microfluidic pumps. In addition, targets are concentrated and purified simply by passing the sample solution over the beads. Beyor et al. later simplified the technique by eliminating the need for recirculation, instead leveraging the pulsatile flow of the microfluidic pumps by incorporating rapid 'flutter' steps followed by a standard 3-step pumping cycle to force the sample solution through the beads multiple times [61]. This method is well-suited for integration in the proposed microsystem because it will facilitate the manipulation of beads for specific capture of mRNA-detection probes from a complex cellular background in addition to immobilization for downstream genetic analysis.

By integrating the sample and bead manipulation necessary for performing the TRAC protocol with PCR amplification and post-PCR capture inline injection, the proposed fully integrated microdevice shown in Figure 3.3 will be able to perform complete gene expression analysis. Magnetic beads are pumped into the microdevice and immobilized using an external magnet to create a localized capture region on the microdevice. The cell lysate sample is pumped into the device and mixed with a pool of fluorescently labeled detection probes and buffer by alternating inputs using a bus valve. The probes are specifically hybridized with the target mRNA in the sample by heating the hybridization chamber, after which the solution is driven through the fluidized bed of magnetic beads using pneumatic microvalve pumping. The oligo(dT)-coated beads capture the mRNA-probe hybrids, and the bead solution is then manipulated hydrodynamically to perform stringency wash steps. An external magnet is used to immobilize the beads while cell debris and excess probes flow out to the waste well. The detection probes are subsequently released in deionized water by heating and the eluate is pumped into the PCR chamber. The PCR reagents are loaded into the capture well and pumped into the PCR reactor to combine with the template for amplification, as previously demonstrated by Liu et al. After thermal cycling, the amplified double-stranded probes are electrophoretically driven through an oligonucleotide-modified capture gel where they are bound and concentrated into a narrow plug prior to injection into the separation channel for CE and detection as previously demonstrated. Because all the analytical steps are performed on a single device, transfer losses are minimized and the results of the analysis are highly quantitative. As a result, this compact microdevice will enable sensitive analysis from a complex sample on a portable and automated platform, decreasing both time and costs required for analysis, and could therefore benefit cancer detection for point-of-care testing applications.

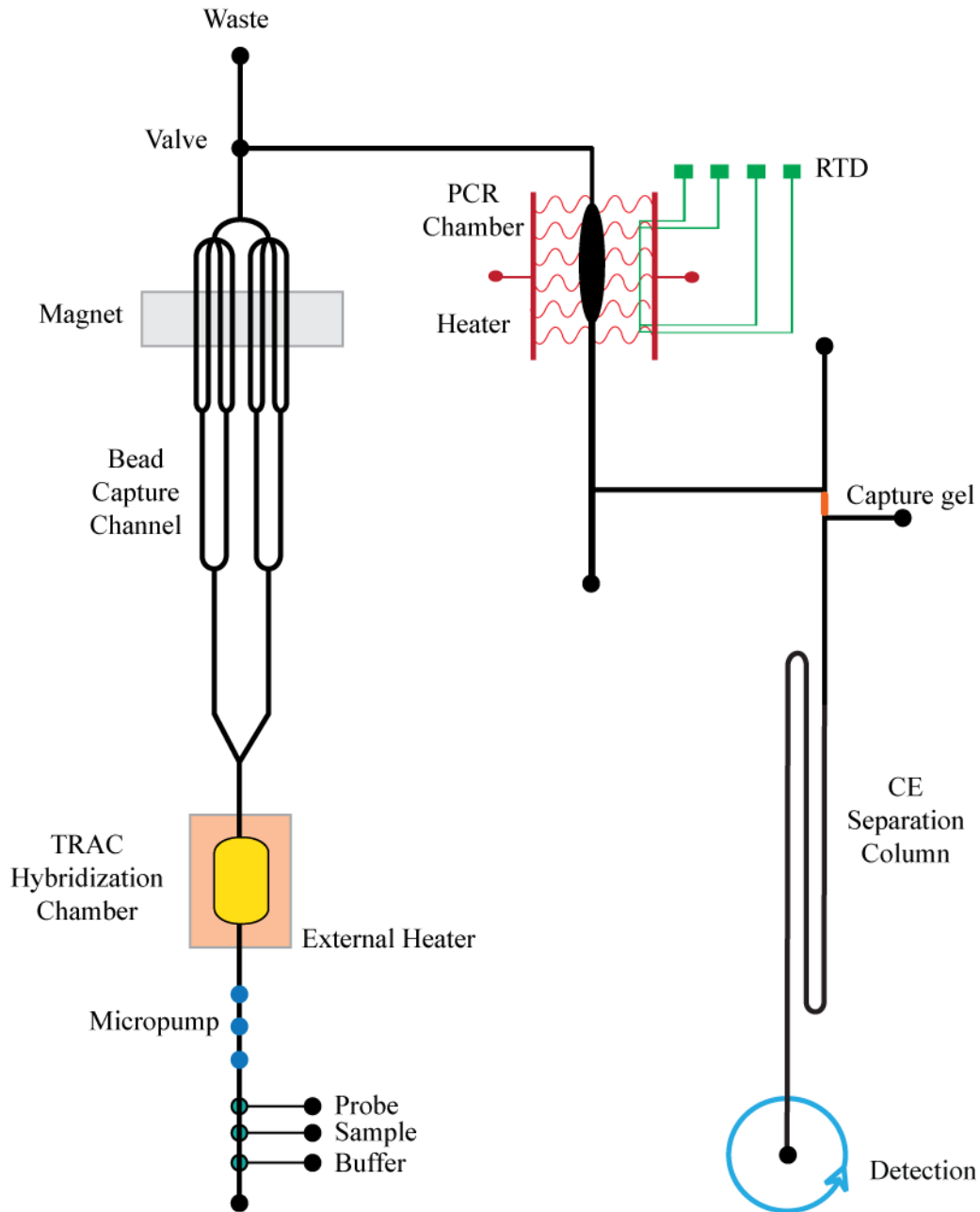


Figure 3.3. Fully integrated microdevice for gene expression analysis. A raw cell lysate sample is pumped into the microdevice and hybridized with a pool of fluorescently labeled detection probes. A fluidized bed of oligo(dT)-coated magnetic beads is used to capture the resulting mRNA-probe hybrids, and excess probes and cellular debris are washed away. After release from the beads, the detection probes are transferred to the PCR reactor for amplification. The amplified probes are then driven electrophoretically into an oligonucleotide-modified photopolymerized capture gel for clean-up and concentration, enabling efficient injection into the CE channel for high-performance separation and detection.

3.4 High-Throughput Analysis Systems

While the extensive integration enabled by the microdevice discussed above already shortens the analysis time considerably, the ability to analyze several samples in one run by increasing the throughput of the microdevice would also facilitate this goal significantly. High throughput devices decrease the overall cost of analysis by handling several samples simultaneously as well as by utilizing less sample and reagents. In addition, increased parallelism is valuable in that it makes maintaining consistent experimental variables across several samples possible. This is especially important for comparing results obtained from analysis of different samples. In particular, samples extracted from suspected tumors are often compared with a “normal” control. By including more than one analysis system on the microdevice, gene expression patterns from two different tissues can easily be contrasted for rapid detection of abnormality.

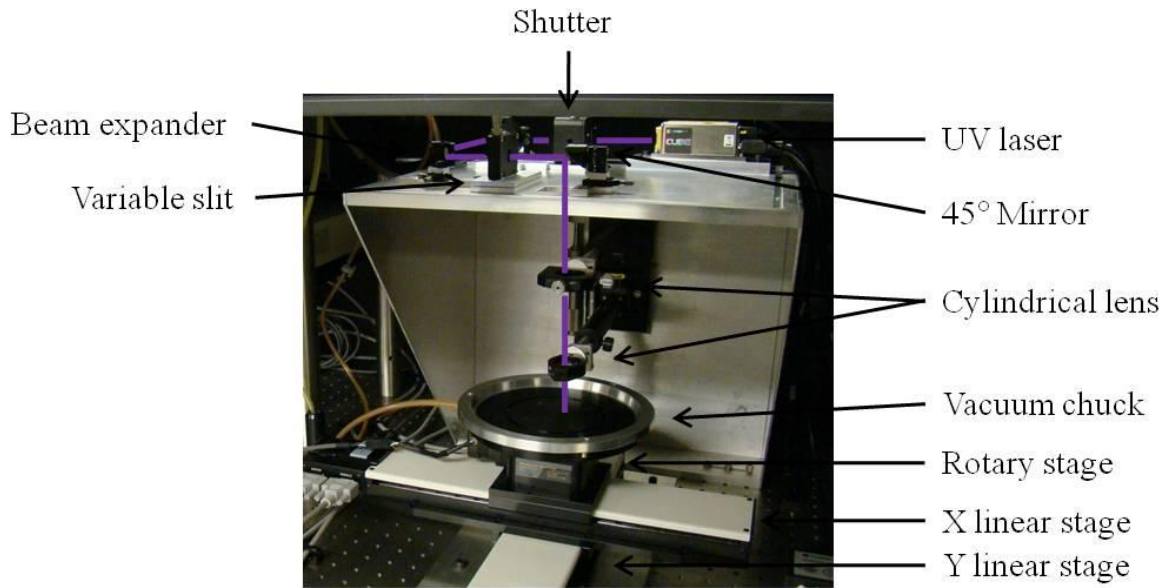
High throughput CE arrays have previously been demonstrated for several applications. A 96-lane capillary microdevice was developed by Paegel et al. in 2002 to perform highly parallelized sequencing [58]. The device featured 48 doublet structures, arranged radially on a 150-mm glass wafer, each consisting of two electrophoretic separation channels with independent sample reservoirs and common cathode and waste wells. Multichannel microdevices for forensic applications have also been reported. These devices contain arrayed microfluidic genetic analyzers that integrate all the functions needed for STR analysis, including PCR reactors with microfabricated RTDs and heaters for thermal cycling, coupled with inline injectors and capillary electrophoresis channels [138]. However, the number of lanes attainable on one microdevice has thus far been limited to 4. Aside from practical limits on the dimensions of the substrate wafer, the main obstacle to increasing this number is the length of time required to prepare the microdevice for analysis.

The photopolymerization of the capture gel matrix plays a critical role in achieving high-performance analysis on-chip by enabling a highly efficient injection that results in increased sensitivity of detection and improved resolution of the separation. However, the exposure system used for the photopolymerization process has thus far limited its applicability to a few channels. In the experiments previously described, photopolymerized gels plugs were created by aligning the polymer solution-filled channel with a photomask and exposing it to UV light supplied by a Mercury arc lamp installed on a microscope. Using this method, the exposure time required for complete polymerization was 5-10 min per plug. Because each channel had to be irradiated individually, exposing a 96-lane device would require an impractical amount of time. In order to allow high-throughput microdevices to achieve their potential for parallel analysis, new methods are needed that will remove this limiting step by allowing the formation of the capture plugs more rapidly.

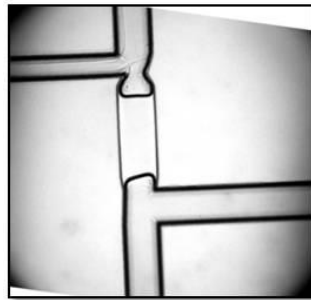
To address this problem, a UV exposure system for rapid and automated photopolymerized gel plug formation is proposed. The instrument utilizes a 375 nm diode laser to photoinitiate polymerization in each channel of the microchip in a stepwise manner (Figure 3.4). First, a system of high precision motorized linear stages aligns a channel of the microdevice with the focused laser beam. Then, the laser beam spot is used to form a gel plug inside the channel. The spot is shaped by first expanding the beam and imaging a rectangular variable slit, followed by focusing using a pair of cylindrical lenses to obtain a uniform rectangular spot. After polymerization is initiated, the laser shutter blocks the beam, and the stage moves to align the laser with the adjacent channel in order to form the next gel.

The motion of the stages is fully controlled by a custom program that outputs coordinates for the correct gel plug locations based on the geometry of the microdevice. As a result, after initial loading of the monomer solution, all the gel plugs necessary for a multi-channel microdevice with integrated affinity capture are formed in an automated manner.

This new method for generating high resolution photopolymerized gel plugs offers several advantages. First, the generation of capture plugs is rapid and fully automated. The high precision axis system reduces polymerization time by allowing simultaneous and coordinated motion of axes as well as specification of move parameters such as velocity and acceleration. Additionally, the use of a laser as the power source generates a small spot with high power density at the wavelength useful for polymerization. Because the laser power is easily tuned, different monomers and photoinitiators can be employed for a variety of applications. Finally, the instrument permits the generation of a range of plug sizes. In contrast to flood exposure through a mask which limits the feature size due to light diffraction at the edge of opaque features, polymerized regions defined by a laser beam can be much smaller. Capture plugs can also be formed by direct write lithography by moving the focused 5 μm beam to form gels in a variety of shapes and sizes. Preliminary experiments with this instrument have enabled the production of 150- μm gel plugs, and this size can be further reduced by optimization of the polymerization variables. For example, laser patterning of 50- μm gel membranes has been previously reported by Song et al. using a similar technique [161]. The ability to generate short gel plugs is extremely valuable for high performance on-chip separations since resolution is directly related to the injected plug size. Based on the instrument's promising results in producing small capture gels in a rapid and automated manner, it is expected to play a valuable role in implementing arrays of high-quality integrated capture-CE microanalyzers for high-throughput analysis of samples.



B



C

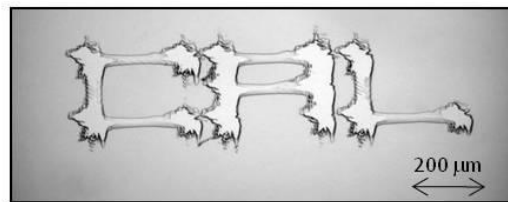


Figure 3.4. High-throughput automated capture gel formation. (A) The beam from a 375 nm UV laser is expanded, shaped and focused for initiating polymerization inside a microdevice. A system of high precision stages is used to control the alignment of the channels with the beam. (B) A 150 μm photopolymerized capture gel plug generated by the instrument. (C) This approach enables high-resolution patterning by direct-write lithography.

3.5 Conclusions

Cancer research significantly benefits from the ability to probe gene expression with high sensitivity. Because of the heterogeneous nature of tumors, multiplex transcript analysis is well-positioned to shed light on the extensive pathways that characterize progression of the disease. In particular, the identification of comprehensive expression patterns involved in pathogenesis can be used for efficient cancer detection. The novel microdevices presented here expand the potential of microfabricated CE devices as a platform for expression-based assays by facilitating high-sensitivity and quantitative transcript analysis from a complex sample. The seamless integration of temperature sensors and integrated heaters for PCR amplification, pneumatic PDMS valve structures for robust fluidic containment and pumping, and gel capture matrices for sample purification and concentration, enable a fully integrated microdevice with all the functionality necessary for a truly “sample in-answer out” point-of-care diagnostic. By virtue of reduced sample and reagent consumption as well as shortened analysis time, this versatile assay platform is promising as an affordable diagnostic tool for widespread use in the clinical setting, and could play an important role in reducing cancer mortality by facilitating early detection.

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