Title
High-Performance Integrated Genetic Analyzers for Forensic DNA Typing

Author
Liu, Peng

Publication Date
2009

Peer reviewed|Thesis/dissertation

High-Performance Integrated Genetic Analyzers for Forensic DNA Typing

by

Peng Liu

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 Tejal A. Desai

Fall 2009

The dissertation of Peng Liu, titled High-Performance Integrated Genetic Analyzers for Forensic DNA Typing, is approved:

Chair _____ Date _____

_____ Date _____

_____ Date _____

University of California, Berkeley

High-Performance Integrated Genetic Analyzers for Forensic DNA Typing

by Peng Liu

Abstract

High-Performance Integrated Genetic Analyzers for Forensic DNA Typing

by

Peng Liu

Joint Doctor of Philosophy in Bioengineering

University of California, Berkeley and University of California, San Francisco

Professor Richard A. Mathies, Chair

Microfabrication technology offers great potential for the integration of all steps of forensic DNA typing onto a single microdevice. This integration should enable rapid, low-cost and reliable short tandem repeat (STR) analysis not only in forensic laboratories but also at crime scenes or other relevant point-of-analysis. As a first step towards making on-site STR typing possible, I developed a microdevice consisting of a 160-nL polymerase chain reaction (PCR) chamber and a 7-cm capillary electrophoresis (CE) channel and a portable instrument for its operation. A four-plex mini Y STR typing system was constructed for testing the capability of this microsystem for forensic STR typing. The successful analyses of casework and mixture samples validate the concept of forensic STR typing on a portable microfluidic system.

To critically evaluate the capabilities of this portable system and the feasibility of DNA typing at a crime scene, real-time DNA analyses using a 9-plex autosomal STR typing system on a modified PCR-CE microdevice containing a co-injection structure for fragment sizing calculation were carried out at a mock crime scene. Blood stain collection, DNA extraction, STR analysis on the PCR-CE microsystem, and a DNA profile search against a mock CODIS database were successfully conducted within 6 hours of crime scene arrival. This demonstration establishes the feasibility of real-time DNA typing at a crime scene or other point-of-care situations.

Finally, to achieve a total integrated analysis system for real-time STR typing, an up-front sequence-specific DNA extraction and concentration method using magnetic beads was developed and incorporated into the PCR-CE microdevice. Fragmented genomic DNA was hybridized with capture probes and immobilized onto magnetic beads via streptavidin-biotin binding in microchannels. The bead-DNA conjugates were then transported to a PCR reactor for amplification followed by inline injection using a novel capture concentration method for CE separation. This fully integrated system significantly advances the forensic DNA typing by providing a high-performance platform with sample-in-answer-out capability for real-time human identification.

Contents

List of Figures

List of Tables

Acknowledgement

There is a long list of people to whom I want to sincerely express my appreciation for their help during my life in Berkeley. This work would never have been possible if it were not for the help I have received from the faculty, collaborators, and my colleagues.

I would like to start by thanking my advisor and committee chair Prof. Richard Mathies, who gave me the freedom of pursuing my research interest and the support I need to complete my project. It seems my research life at Berkeley was filled with all kinds of deadlines which Rich gave me. As a result, my research was tough in these years. But when I looked back in the end, I found I have achieved more than I could ever imagine and these achievements would never have been possible without Rich's supervision. Thanks should also be given to Mary Hammond for always finding time to help me with so many little things that make my life easier. I would also like to thank my committee members, Prof. George F. Sensabaugh and Prof. Tejal A. Desai for their care and their review of my dissertation.

Thanks to my collaborator, Susan Greenspoon at the Virgina Department of Forensic Science for giving me so many interesting forensic samples to try on my system. Crouse Cecelia and Karin Crenshaw at the Palm Beach County's Sheriff Office deserve special thanks for their help on the crime scene test. Their passions to forensic research helped me believe in how important my research is to our society.

I would like to thank everyone in the Mathies lab. First is our lab scientist, Dr. James R. Scherer. Without the instruments he built for us, all my work would have been impossible. I also learnt a lot from Jim on optics, instrument design, characterization, and evaluation. Next is Stephanie Yeung, who is so considerable and friendly. When I first started to work on this project, I knew nothing about forensic STR typing. Stephanie taught me many basic concepts and techniques. She also gave me countless suggestions and helps throughput my research in Berkeley. Nate Beyor is the first student I worked with in this lab. He taught me how to use all the basic equipments. Eric Chu helped me on microfabrication and always let me realize my microfabrication skill needs to be polished more. Prof. Shin provided me the mini-Y STR typing system and Tae Seok Seo helped me on the synthesis of energy-transfer dyes. Their helps are the key to the success of my first publication. In the last year, I was working with Numrin Thaitrong. She not only helped me a lot on the research, but also let me know many interesting things happened in the lab. Yong Zeng, Chaoyong Yang, and Xiujin Li are the friends in my life. I won't forget all the fun we have together. Great thanks also go to all the other members of the Mathies group. Their kind help made my life in Lewis Hall happy.

I also thank Eric Granlund in the College of Chemistry machine shop for making many of my designs become real, and Henry Chan in the Chemistry electronics shop for always giving me timely help during troubleshooting of the instruments. Thanks should also be given to the staff members in the Department of Bioengineering. Their efforts made my work at Berkeley happy and convenient.

Special gratitude goes to my parents Defa Liu and Xiuyun Fan. I know they are very proud of me and I didn't let them disappointed. Finally, I would like to thank my wife Xiaofang. We have been separated for six years, which was the hardest time for both of us. But finally we overcame it. I want to say to my wife: we did it, and separation will never happen again to us.

This dissertation is dedicated to
my wife & my parents

Chapter 1

Forensic DNA Typing

1.1 History of Forensic Human Identification

The technologies for forensic human identification have evolved with our understandings of our own bodies. While visual identification of human faces and bodies seems the most straightforward strategy to recognize and differentiate persons, it is not uncommon for eyewitnesses to point out the wrong person during forensic investigations. In 1883, a French police officer Alphonse Bertillon devised a scientific means for recording human physical traits.[1] But this identification system was only accepted for thirty years, and it soon made way to another more reliable method, fingerprinting.

The modern concept of fingerprinting was developed in the 1880s by Henry Faulds. Inspired by the human finger imprints on ancient artifacts while in Japan, Faulds published his idea of using fingerprints in forensic investigations in the scientific journal *Nature*.[2] Since then, the use of fingerprints has been established as a standard for human identification around the world. Although fingerprints are effective for differentiating individuals, their low availability in crime cases keeps researchers constantly looking for alternatives. Many methods, such as those based on blood ABO groups and human leukocyte antigen (HLA) differences, have been developed, but they can only function as supplementary tools because of the limited variability in the population. Forensic human identification has thus been dominated by the fingerprinting methodology for almost a century.

The revolution in forensic casework investigations began in 1985 when Alec Jeffreys and coworkers turned their eyes to DNA for human identification.[3, 4] They discovered that some regions of the human genome contain a number of repetitive sequences which are arranged next to each other. Although these tandem repeat sequences are usually the same between persons, the repeat number could be different from one individual to the other. In 1986, DNA typing was successfully employed to analyze a blood sample from Colin Pitchfork and match his DNA profile to that of a semen sample recovered from two murders committed in England in 1983 and 1986.[5] Since then, the technologies and forensic DNA markers for DNA typing have moved at a breathtaking pace. Within a short period of time, DNA analysis has been accepted as an indispensable and routine method by most forensic laboratories. Large DNA databases have become available for forensic investigators to search for DNA profile matches, and many 'cold' or questionable cases have been re-examined using DNA typing. The use of DNA typing in several well-known legal cases, such as the O.J. Simpson case[6] as well as President Clinton's scandal,[7] have also helped DNA typing attract great public interest. All these remarkable achievements have announced the advent of the DNA era in forensic human identification.

1.2 Forensic DNA Markers

Knowledge of human genome sequences reveals that most of the genetic variations are in the "noncoding" sequences of the human genome because mutations in these regions can usually be transmitted to the offspring in absence of selection pressure during evolution. [8] An important percentage of the noncoding DNA consists of repetitive sequences that can be divided into two classes: tandemly repetitive sequences and interspersed elements. The forensic genetic markers currently in use are mainly located in the tandem repetitive sequences because this variation can be readily examined via DNA lengths rather than DNA sequences. Other genetic variations,

such as single nucleotide polymorphisms, also play increasingly important roles in forensic DNA typing, but are generally more expensive and time consuming.

1.2.1 Variable number of tandem repeats

The forensic DNA markers that Alec Jeffreys discovered are known as variable number of tandem repeats (VNTR).[8] Since the lengths of the repeats are in the range of approximately 10-100 bases and the total lengths are 500 bp to 20 kb, these DNA regions are also called minisatellites to distinguish them from the more common regions of satellite DNA, which are 20-100 kb in length. The method that Dr. Jeffreys developed for examining these genetic variations was restriction fragment length polymorphism (RFLP). Briefly, the analytical procedure is as follows: DNA samples are first digested into small fragments by specific restriction endonucleases, such as *Hinf*I and *Hae*III, and then separated based on the fragment sizes using agarose gel electrophoresis. In a subsequent Southern blotting step, the separated DNA bands are transferred from the gel onto a nylon membrane, followed by hybridization with single-stranded radioactive or chemiluminescent DNA probes. After binding and washing, the pattern of the DNA bands on the membrane can be visualized using an x-ray film and analyzed for human identification. The main advantage of RFLP is its high discrimination power. For example, using only a single probe, the match probability is estimated to be $< 3\times10^{-11}$ and two probes together increase this value to $< 5\times10^{-19}$.[4] However, RFLP suffers from some critical disadvantages: RFLP requires high molecular weight, intact double-stranded DNA samples in order to produce high-fidelity digested fragments using restriction enzymes. The analytical process is time consuming, usually requiring about 1 week with chemiluminescent probes and 6-8 weeks with radioactive probes, and it is not easily automated. Because of these drawbacks, RFLP is used nowadays only in a few forensic laboratories, particularly for paternity testing analysis.

1.2.2 Short tandem repeats

Over the last decade, VNTRs has been replaced by another type of DNA marker for forensic DNA typing: short tandem repeats (STRs) or microsatellites.[9, 10] STR markers are estimated to account for 3% of the human genome, with a frequency of one per 10,000 nucleotides.[11, 12] STRs have 2-7 bp long repeat units and their total length is significantly shorter than VNTRs, usually between 100 and 500 bp. Table 1.1 shows the comparison between VNTRs and STRs for forensic DNA typing. The advantages of using STRs for DNA typing over VNTRs generally stem from their short total lengths, which can be readily amplified at multiple loci from minute amounts of DNA as well as degraded samples in a single polymerase chain reaction (PCR) as shown in Figure 1.1. After PCR, electrophoretic separation coupled with fluorescence detection is employed, providing STR assays with high sensitivity and high speed. Nowadays, STR analysis has been widely accepted as the gold standard for DNA typing and many countries have established DNA databases based on STR loci. In the United States, the national DNA database is known as Combined DNA Index System (CODIS), and as of August 2009, the National DNA Index (NDIS) of the CODIS contains over 7,344,364 offender profiles and 281,068 forensic profiles.[13]

A total of 13 STR loci are employed as the core markers in CODIS, providing a discrimination power of 1 in a trillion among unrelated individuals.[14, 15] Table 1.2 lists the detailed information of these 13 loci. Multiplex PCR kits which can co-amplify all 13 loci in a single reaction along with the amelogenin sex-typing marker and two additional STR loci have

become commercially available from two US companies: Promega (Madison, WI) and Applied Biosystems (Foster City, CA).

Table 1.1. Comparison of VNTRs and STRs for forensic DNA typing

Characteristic	VNTR using RFLP	STR using PCR
Analysis time	~1 week with chemiluminescent probes	~ 1 day
DNA amount	50-500 ng	0.1-1 ng
DNA quality required for analysis	High molecular weight, intact DNA	Can be degraded DNA
Mixture sample analysis	Yes, single-locus probe only	Yes
Automation	No	Yes
Power of discrimination	~1 in 1 billion with 6 loci	~1 in 1 billion with 8-13 loci

Table 1.2. Information of the CODIS 13 STR loci

Locus Name	Chromosomal Location	Repeat Sequence	GeneBank Accession	Range of repeats at alleles
D3S1358	3p21.31	[TCTG][TCTA]	NT_005997	8-21
TH01	11p15.5	TCAT	D00269	3-14
D21S11	21q21.1	Complex [TCTA][TCTG]	AP000433	12-41.2
D18S51	18q21.33	AGAA	L18333	7-39.2
D5S818	5q23.2	AGAT	G08446	7-18
D13S317	13q31.1	TATC	G09017	5-16
D7S820	7q21.11	GATA	G08616	5-16
D16S539	16q24.1	GATA	G07925	5-16
CSF1PO	5q33.1	TAGA	X14720	5-16
vWA	12p13.31	[TCTG][TCTA]	M25858	10-25
D8S1179	8q24.13	[TCTA][TCTG]	G08710	7-20
TPOX	2p25.3	GAAT	M68651	4-16
FGA	4q31.3	CTTT	M64982	12.2-51.2

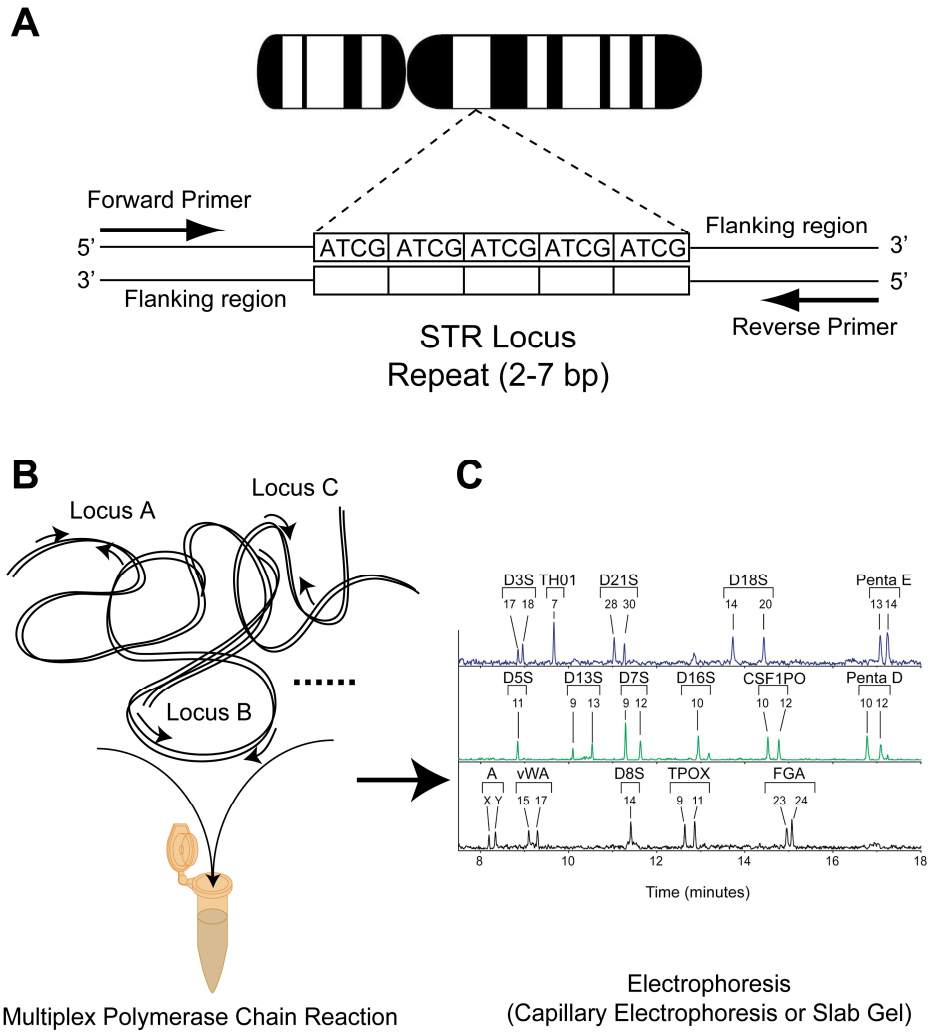

Figure 1.1. Schematic of short tandem repeat typing.

(A) STR locus contains 2-7 bp repeat units and the total length is about 100-500 bp. By using a forward and a reverse primer on the flanking region, the repeat sequences can be amplified for DNA typing. (B) In a single PCR reaction, multiple STR loci can be equally co-amplified by carefully designing the primers and optimizing the primer concentrations. (C) Following PCR, the amplified fragments are separated using capillary or slab gel electrophoresis to determine repeat numbers in each STR locus with the aid of internal lane standards and allelic ladders, generating a unique barcode for each individual. To accommodate up to 16 loci in the size range of 100-500 bp, multi-color dye labeling of the primers is usually employed.

STR loci located in the Y chromosome also play an important role in forensic investigations, enabling male specific DNA typing.[16] As most violent crimes, especially sexual assault cases, are committed by males, Y-chromosome STR typing is particularly useful for extracting Y-STR profiles from a mixture with high female DNA background. The drawback of Y-STR typing is, however, the limited discrimination power due to the lack of chromosome reassortment and recombination in Y STR loci.[16] In 2003, the Scientific Working Group on DNA Analysis Methods (SWGDAM) recommended the use of a total of 11 Y-chromosome STR loci for male specific typing, as listed in Table 1.3. Some commercial Y-STR kits (PowerPlex® Y and Yfiler™) are also available now, leading to the extensive application of Y-STR typing in forensic caseworks.[17-19]

Table 1.3. The information of SWGDAM-recommended Y-STR loci

Locus Name	Position (Mb)	Repeat Sequence	Range of repeats at alleles
DYS393	3.04	AGAT	8-16
DYS19	9.44	TAGA	10-19
DYS391	13.41	TCTA	6-13
DYS437	13.78	TCTA	13-17
DYS439	13.83	AGAT	8-15
DYS389I/II	13.92	TCTG TCTA	10-15/24-34
DYS438	14.25	TTTTC	8-12
DYS390	16.52	TCTA TCTG	18-27
DYS385 a/b	20, 20.04	GAAA	7-25
DYS392	21.78	TAT	7-18

1.2.3 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are single-base variations in the human genome, that are highly abundant (1 in every 1000 bases) and thus likely to play a role in human identification.[20-22] SNPs are appealing to the forensic community for several reasons.[23] First, SNPs can be analyzed in short amplicons (<100 bp in length), allowing a higher level of PCR multiplexity and resulting in more successful analysis of degraded DNA. Second, since no size-based separation is needed for SNP detection, high-throughput technologies such as microarrays can be employed, and data analysis can be fully automated.[24] Third, SNP markers can be used to predict ethnic origins and certain physical traits, which are particularly useful for searching for suspects during forensic investigations. However, some intrinsic disadvantages of SNP analysis limit its application in forensics and make it unlikely that SNP analysis will replace STR typing in the near future. SNPs are bi-allelic markers, which means they have only two possible alleles and three possible genotypes. As a result to achieve the same discrimination power as that of STRs, many more markers are needed, leading to difficulties in simultaneously amplifying enough SNP markers from low amounts of DNA. Additionally, the interpretation of mixture

samples using SNPs is challenging because it is difficult to differentiate a true heterozygote from a mixture of two homozygotes or a heterozygote and a homozygote. Despite these drawbacks, SNP analysis can still be useful in providing vital supplementary information to forensic investigations.

Single-nucleotide polymorphisms located in the human mitochondrial DNA are another powerful tool for human identification.[25] Although it shares the same limitations as the Y-STR loci, such as less diversity caused by uniparental inheritance through matrilines, the advantage of high DNA copy numbers (200 - 1700 copies) in a single cell makes mitochondrial DNA typing particularly useful in the analysis of samples that are severely damaged, or contain low DNA amounts (such as hair shafts). The common mitochondrial DNA typing method is to directly sequence two hypervariable regions within a control region, known as hypervariable segments I and II (HVSI, HVSII). Since the cost of DNA sequencing has been reduced dramatically, mitochondrial DNA typing has become increasingly available in forensic laboratories.[26]

1.3 Procedure for STR Typing

Short tandem repeat analysis has become the gold standard for forensic human identification due to its ability to generate highly distinctive profiles from minute amounts of DNA.[9, 10, 27] However, due to the precipitous gap between the increasing numbers of samples submitted for examination and the limited processing capability of current forensic laboratories, escalating backlogs are accumulated in our nation's state and federal crime labs. Furthermore, a unique challenge faced by forensic scientists is the diverse sample quality in STR analysis, including low-copy-number (LCN), degraded, and mixture DNA samples, which is the major cause of failure in DNA typing.[28-30] To address these issues, the current process of forensic STR analysis should be thoroughly reviewed to determine possible improvements.

A typical STR analysis procedure in a crime laboratory includes three steps: sample preparation, PCR amplification, and DNA separation. Forensic DNA samples come from a variety of sources, such as blood, bone, urine, semen, saliva, and hair, and are usually exposed to the environment for various periods of time, potentially resulting in sample contamination and degradation.[31] As a result, DNA must be extracted from background materials, which may contain PCR inhibitors, and put into a suitable solution format at an appropriate concentration. Historically, DNA extraction methods used in forensic laboratories include phenol-chloroform extraction[32] and CHELEX.[33] However, since these methods usually include multiple centrifugal steps and employ toxic organic solutions, the extraction process is hard to automate and scale up. Recently, solid-phase extractions with formats of filters, columns, and beads have gradually been accepted by forensic scientists due to their advantages of simple operation and compatibility with robotic systems,[34-36] but further validation of these methods for forensic casework is still underway.

For the step of STR amplification, the majority of domestic forensic laboratories currently use the STR multiplex kits manufactured by Promega (PowerPlex® 16 and 16 BIO, PowerPlex® 1.1, 2.1 and 1.2) and Applied Biosystems (AmpFLSTR® Profiler®, Cofiler®, and Identifiler®).[37-39] The PCR performed in a thermocycler takes about 3 hours, primarily due to the slow thermal ramping of these bulk instruments. Therefore, a thermocycler with faster temperature ramping capability is desired to improve the turn-around time of STR analysis.

Following PCR, DNA separation and detection is carried out using electrophoresis coupled with fluorescence detection. The most commonly utilized platforms are the ABI 310, 3100 and 3100-Avant Capillary DNA Sequencer instruments and the Miraibio FMBIO® II, IIe and III plus fluorescent image analysis systems.[40] When using an ABI 3100 Capillary DNA Sequencer with 16 capillaries, which provides the highest throughput available for forensic scientists, the analysis time is approximately 30 to 40 minutes for a single separation and several hours for analysis of a single tray of 96 samples. Thus, a single instrument may not provide sufficient throughput for a forensic laboratory for routine work.

It is evident that the forensic community would greatly benefit from the automation, miniaturization, and integration of the DNA typing process, because: (i) automated analysis reduces the sample turn-around time, saves labor costs, and eliminates the risk of sample contamination and mix-up; (ii) miniaturized instruments consume less sample and reagents, speed up analysis, and may enable point-of-care DNA typing; (iii) an integrated process allows efficient sample transfer between analytical steps, leading to increased sensitivity and reliability for challenging sample profiling.

1.4 Scope of the Dissertation

Over the past two decades, microfabrication technology has demonstrated a great potential to significantly improve the sensitivity, cost, speed, and reliability of genetic analysis.[41, 42] Although forensic DNA typing has a similar analytical process as other genetic analysis, it has not yet fully benefited from the development of microfabrication. The goal of the work presented in this dissertation is thus to develop fully integrated micro total analysis systems for forensic human identification by applying the state-of-the-art in microfabrication technology.

Chapter 2 reviews the current state-of-the-art for microfluidic devices in DNA sequencing, gene expression analysis, pathogen detection, and forensic DNA typing. From this review, it is evident that several key elements, including device materials, temperature control, microfluidic control, and sample/product transport, must be carefully chosen in order to produce a microsystem with high performance.

In Chapter 3, a portable forensic analysis system consisting of a microfluidic device for amplification and separation of STR alleles together with a portable instrument for chip operation is presented. This work is a significant step towards a fully automated portable device that allows rapid STR analyses in a setting outside the forensic laboratory.

In Chapter 4, I further explore the concept of point-of-analysis forensic human identification by developing an improved integrated PCR-CE microdevice and typing method capable of conducting real-time forensic STR analysis. To critically evaluate the capabilities of this portable microsystem as well as its compatibility with crime scene investigation, real-time DNA typing was successfully carried out at a mock crime scene. Sample collection, DNA extraction, STR analysis on the microsystem, and a DNA profile search against a mock CODIS database were conducted within six hours of crime scene arrival. This demonstration establishes the feasibility of real-time DNA typing to identify the contributor of probative biological evidence at a crime scene and for real-time human identification.

To move towards a truly micro total analysis system, I developed a sequence-specific DNA extraction method using magnetic bead capture and successfully integrated this function as

well as the improved post-PCR capture inline injection into the PCR-CE microsystem presented in Chapter 5.

Finally, Chapter 6 concludes this thesis by discussing the possible improvements that can be made to integrated microsystems for STR analysis.

Chapter 2

Integrated Microfluidic Systems for High-Performance Genetic Analysis

Reproduced from “Integrated Microfluidic Systems for High-Performance Genetic Analysis” by Peng Liu and Richard A. Mathies in Trends in Biotechnology, 27, 572-581 (2009), with permission from Cell Press.

2.1 Summary

Driven by the ambitious goals of genome-related research, fully integrated microfluidic systems have developed rapidly to advance biomolecular and especially genetic analysis. To produce a microsystem with high performance, several key elements must be strategically chosen, including device materials, temperature control, microfluidic control, and sample/product transport integration. We review several significant examples of microfluidic integration in DNA sequencing, gene expression analysis, pathogen detection, and forensic short tandem repeat (STR) typing. The advantages of high speed, increased sensitivity, and enhanced reliability enable these integrated microsystems to address bioanalytical challenges, such as single-copy DNA sequencing, single-cell gene expression analysis, pathogen detection and forensic human identification in formats that are enabling for both large scale and point-of-analysis applications.

2.2 Introduction

With the completion of the reference human genome sequence and additional individual sequences in hand,[43-45] even more ambitious goals for future genome-related research come to mind. Exploring the implications of genome variation for speciation, evolution, and disease,[46, 47] studying gene expression and regulation at the single-cell level,[48, 49] as well as improving forensic and clinical genetic analysis,[9, 50] are now on the horizon. Technologies that will enable these advances of genetic analysis must be fast and inexpensive, have high sensitivity, and provide flexible and robust platforms.

While automated genetic analysis has advanced significantly in the past decade through the application of robotics, several intrinsic drawbacks are increasingly evident: (i) The liquid handling limits of robotic analytical techniques usually are in the μL scale, which not only consumes expensive reagents, but also leads to inevitable sample dilution. For example, to analyze a single gene in a cell, we conventionally put it into a working volume of 10 μL, which results in extreme dilution down to $<10^{-18}$ M. This is problematic since the most sensitive systems for DNA detection typically require concentrations in the femtomolar-picomolar range.[51] (ii) During conventional genetic analysis, samples are transferred between multiple instruments, which can cause further sample dilution and loss. For instance, in DNA capillary electrophoresis (CE) analysis, the loaded sample is typically 1-2 μL, but only ~2 nL of this sample volume is effectively injected into the capillary for separation and detection.[40] (iii) Contamination issues become prominent when dealing with low-copy-number or single-cell samples, since the contaminants can overwhelm the real target signals. Current analytical processes which have multiple open sample transfer steps make contamination inevitable.[27] Paradoxically, the final analytical systems for genetic analysis typically do not require a large amount of sample. For example, only 10^6-10^7 molecules are sufficient for CE detection. While robotics provides the macro integration of analytical processes which can address some of the above problems, a fully integrated and automatic system that operates on the nanoliter volume scale would enable improved performance.

2.3 Integrated Microfluidic Systems

Micro-total analysis systems (μTAS) have the potential to overcome all the above problems due to their capability of integrating multiple analytical steps into a single microdevice at the pL-nL volume scale using microfabrication technology. The advantages provided by such a "lab-on-a-chip" system are recognized as high speed, high throughput, low reagent consumption, and the reduction of the instrument size.[41, 42] Moreover, the limited diffusion distances and concentrated reagents achieved by performing reactions in pL-nL structures, substantially increases the sensitivity and the speed of assays.[52] By integrating the analytical process on a single device, we can achieve efficient connections between each functional unit, so that sample loss and dilution is minimized. Additionally, the microfluidic automation eliminates the risk of sample mix-up and contamination. Given all these inherent advantages, fully integrated microfluidic systems are a promising technology.

In this review we will first discuss several important considerations for the design and development of fully integrated micro total analysis systems, before highlighting significant

recent advances in the areas of DNA sequencing, gene expression analysis, pathogen/infectious disease detection, and forensic short tandem repeat (STR) typing.

2.4 Development of Integrated Microdevices

To develop fully integrated microsystems for gene expression and genetic analysis, four elements critically impact process integration: i) device material, ii) heaters and temperature sensors for thermal cycling of reactions, iii) microvalves for partitioning analytical steps, and iv) sample/product transport between analytical steps. The choices that are made in each of these areas will determine the challenges and successes that are achieved in the integrated analytical system.

2.4.1 Device materials

The choice of device materials is important for the development of integrated microsystems, since the design, fabrication and operation of the device are heavily dependent on the properties of the substrates. Glass by far remains the most extensively used substrate for implementing integrated microfluidic devices for genetic analysis due to its particular advantages, including high dielectric strength, optical transparency for detection, and mature surface chemistry manipulation.[53, 54] The primary disadvantage of glass is its high material cost and more complex fabrication. However, it is also worthwhile to note that disposable glass microchips can be made at low cost when manufactured in high volume.[55] Plastics and elastomers, such as poly(methylmethacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS), have also been successfully utilized in microsystems and are becoming increasingly popular.[56, 57] In contrast to glass, the simple fabrication and low material cost make plastic and elastomers better choices for disposable devices. However, these materials have some fundamental challenges due to their lack of facile surface modification techniques, inherent fluorescence/Raman background, low glass transition temperature, and incompatibility with metal microfabrication for sensor integration.

2.4.2 Temperature control

Gene expression and genetic analyses usually include DNA or RNA amplification steps, which require rapid and accurate temperature control for thermal cycling of reagents in microreactors. Many temperature control methods, including contact and non-contact heating, have been successfully demonstrated on microdevices. Contact heating methods include external heaters attached to the chip surface, such as Peltier heaters, and microfabricated thin film heaters made of either Ti/Pt,[58] aluminum,[59] or Indium Tin Oxide (ITO).[60] Non-contact heating can be realized by infra-red (IR) irradiation.[61] While these heating systems demonstrate similar performance in a laboratory setting, contact heating is more suitable for point-of-care applications due to its inherently small size and facile integration and operation.

2.4.3 Microvalves for partitioning analytical steps

In a fully integrated microfluidic system which contains several analytical steps, microvalves are essential parts for physically separating each functional unit. Microvalves can be roughly categorized into four different groups: i) active mechanical, such as electromagnetic,[62] piezoelectric,[63] and pneumatic valves,[64, 65] ii) active non-mechanical, including phase change material valves,[66, 67] iii) passive mechanical, such as check valves,[68] and

iv) passive non-mechanical microvalves, including hydrophobic[69, 70] and gel valves.[71] The selection of a particular microvalve depends on the following considerations: whether it is normally in closed or open mode, its dead volume, power consumption, pressure resistance, reusability, insensitivity to particle contamination, fabrication complexity and cost. Generally speaking, active mechanical microvalves have the best performance and are the most commonly used in microsystems. However, in a given application other simple passive valves may be more suitable. For example, in an integrated PCR-CE microdevice, the interface between the viscous separation matrix and the PCR solution can act as a barrier to restrain the PCR solution in the reactor during thermal cycling.[58] While such a passive mechanical valve is uncontrolled and not resistant to high pressure variations within the channels, it can be sufficiently reliable for this situation with care.

2.4.4 Sample/product transport

The integration of a whole analytical process on a single device is much more than the simple combination of several microfabricated units. Efficient and reproducible sample/product transport between functional units is the key to seamless integration that demonstrates the previously mentioned advantages of sensitivity, reproducibility, and reliability. Methods to transport samples within a device include: i) transport by active pumps,[72] ii) transport by electric field,[73] iii) transport vehicles, such as DNA capture and transport using magnetic beads,[74, 75] and iv) capture at sample destination by filters,[76] capture gel,[77] or solid phase columns.[78] The pump and electric field methods are simple, but they can require delicate timing optimization. Especially, when volume change occurs during transport, sample will be discarded or diluted. In contrast, the carrying and capture methods are more efficient and reliable, particularly when it is necessary to make a large change in sample volume or exchange of buffers. A good example of the challenges arising from sample transport is provided by microchip capillary electrophoresis (μCE). Nearly all the μCE systems developed so far employ the classical cross-injector to form narrow sample plugs for CE separation.[42] When excess sample is supplied by off-chip preparations, this method works well in establishing a significant amount of analyte in the cross-injection region. However, in a fully integrated microsystem, such as integrated PCR-CE devices,[53, 58, 79] this simple electrokinetic injection can be problematic because the amount of sample provided by the nL-scale device might not be sufficient to provide a good injection.

To solve this problem, researchers have developed various inline methods for sample preconcentration and injection. For example, Ueberfeld et al. developed a DNA sample loading method using carboxyl-modified magnetic beads for DNA capture in chip-based electrophoresis (Figure 2.1a).[74] As an alternative, Long and coworkers utilized a solid-phase extraction column coupled with a CE separation channel for sample purification, concentration, and injection (Figure 2.1b).[78] More recently, the Mathies group developed post-PCR sample capture and inline injection methods using an oligo- or streptavidin-modified capture gel, which purified and injected PCR products with near 100% efficiency (Figure 2.1c and 2.1d).[80, 81] Integrated PCR-CE microdevices with such simple inline injectors have been successfully developed and demonstrate significantly enhanced sensitivity (10-20 fold) and reliability because of their quantitative transfer capability.[80]

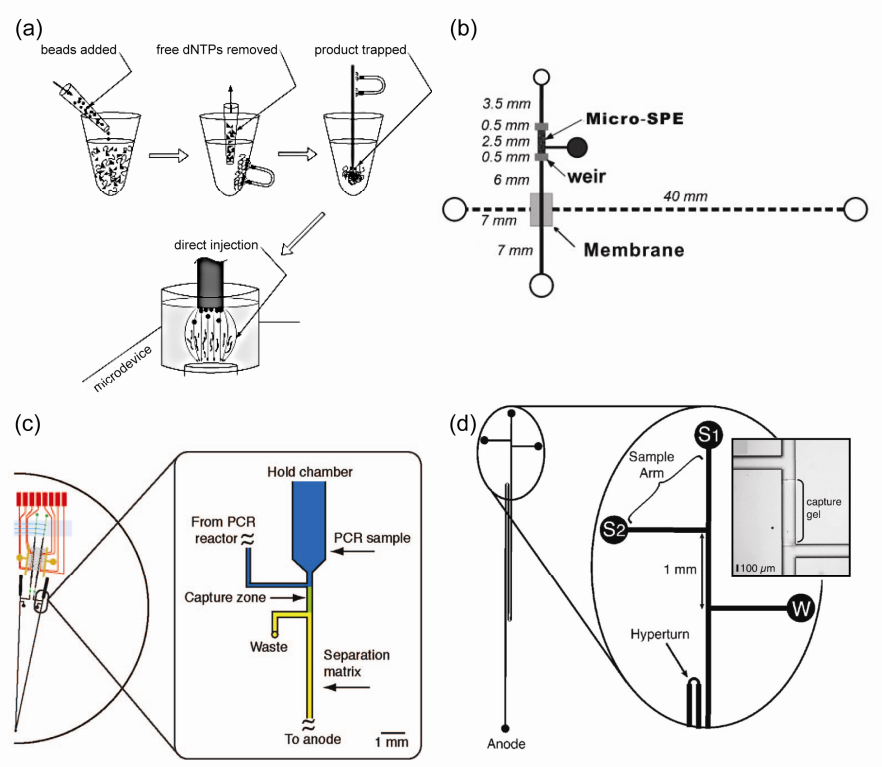

Figure 2.1. Methods for sample preconcentration and inline injection.

(a) Schematic of DNA loading procedure using paramagnetic beads for chip-based electrophoresis. Paramagnetic beads are added to a tube and adsorb the DNA sample. Then, the beads are captured on a magnetized wire and transported to the microchip, where the wire is used as an electrode for direct electrokinetic injection of DNA. (Adapted with permission from Reference 74) (b) An integrated PDMS microchip with a solid-phase extraction column for sample preconcentration, injection and separation. A nanoporous membrane is sandwiched between two PDMS substrates to isolate the upper SPE channel (solid line) and the lower CE separation channel (dashed line). Following sample purification performed in the upper channel, a voltage pulse is applied to inject the concentrated sample through the membrane to the lower channel for electrophoresis. (Adapted with permission from Reference 78) (c) An integrated PCR-CE microdevice with post-PCR capture and inline injectors. In the enlarged schematic, a capture matrix made of 5% acrylamide/bis gel with covalently linked oligonucleotide capture probes is photopolymerized in a channel (green). PCR products generated in a reactor are injected through channels (blue) and captured on the gel plug. After washing, purified and concentrated PCR products are thermally released into a separation channel (yellow). (Adapted with permission from Reference 80) (d) Schematic of a CE chip integrated with an inline injector. A 900-μm-long streptavidin-gel plug is photopolymerized next to a separation channel. Biotin-labeled PCR products can be captured and injected into the separation channel for electrophoresis. The embedded photograph shows the photopolymerized capture gel in the channel. (Adapted with permission from Reference 81)

2.5 Applications of Integrated Microfluidic Devices

The development of fully integrated microfluidic devices is advancing rapidly and has already led to some significant achievements in the areas of DNA sequencing, gene expression analysis, pathogen detection, and forensic STR typing, which are discussed in detail below.

2.5.1 DNA sequencing

To meet the expanding demands of DNA sequencing, extensive research has been conducted to develop new sequencing techniques and to improve the Sanger sequencing method. Next-generation DNA sequencing techniques are evolving rapidly and have been commercialized into products providing massively high throughput. For example, the 454 GS FLX instrument (Roche Applied Science) generates ~ 1 million reads per run at lengths of 400 bases (http://www.454.com/products-solutions/system-benefits.asp). Solexa technology (Illumina, http://www.illumina.com/downloads/GenomeAnalyzer_ SpecSheet.pdf) and SOLiD™ 3 system (Applied Biosystems, http://www3.appliedbiosystems.com/AB_Home/ applicationstechnologies/SOLiDSystemSequencing/overviewofsolidsystem/index.htm) provide higher throughput (>300 million and 400 million reads per run) with shorter read lengths (75 and 50 bases). Single-molecule sequencing methods, such as HeliScope system (Helicos, http://www.helicosbio.com/Portals/0/Documents/Helicos_SalesSpec.pdf) and sequencing detected by zero-mode waveguides (Pacific Biosciences),[82, 83] have also been successfully demonstrated with performance of 400 million reads at lengths of 30 bases and 4.5 million reads at lengths of 1500 bases suggested in early prototypes.

Sanger sequencing providing long reads (~700 bp) with modest throughput (hundreds of thousands of reads per day) remains the best option for *de novo* sequencing of complex new genomes and low-scale applications because of its long read lengths and flexibility in scale. There is a tremendous opportunity for improvement of Sanger biochemistry to its ultimate molecular limits and to higher throughput using microfabrication technology. Separations of sequencing samples on microfabricated CE devices have already yielded impressive results,[84, 85] such as ultrafast separations of 600 bases in just 6.5 min on a CE chip,[86] and a 768-lane sequencing system for high-throughput analyses.[87] However, the true power of a microfabricated platform lies in its ability to integrate sample preparation with electrophoresis to achieve rapid and low-cost DNA sequencing from minute amounts or even single copies of DNA template.

In 2006, Mathies' group demonstrated a nanoliter-scale microfabricated bioprocessor which integrated all three Sanger sequencing steps, thermal cycling, sample purification, and capillary electrophoresis into a 4-inch hybrid glass-PDMS wafer.[54] This fully integrated system contains a 250-nL reactor for thermal cycling, PDMS micropumps and valves for efficient sample transport, affinity-capture chambers for DNA sequencing product purification, and a 30-cm channel for CE separation. DNA sequencing from only 1 fmole of DNA template was performed in less than 30 min with a 556-base read-length and 99% accuracy. This breakthrough provides an excellent core engine from which further improvements can be made to explore the ultimate sensitivity limit of the Sanger biochemistry - sequencing of a single template molecule. To achieve this goal, Blazej and coworkers developed a gel-based affinity method for DNA capture, concentration, and inline injection that was integrated with on-chip CE.[77] About 30 nL of sequencing sample produced from only 100 attomole of human mitochondrial HVII template could be quantitatively immobilized in a capture gel and inline

injected into a separation channel for electrophoresis. By incorporating such an inline injector into the previous nanoliter-scale bioprocessor,[54] the starting template for a successful DNA sequencing could be reduced 10-fold, from 1 femtomol to 100 attomol – this turns out to be a fundamental advance.

A sensitivity of 100 attomol is important as this is the amount of product that could be easily produced by PCR amplification of a single template molecule.[54] This raises the question whether it is practically possible to amplify a single template and subsequently perform Sanger sequencing. Recently, Kumaresan et al. provided an affirmative answer to this question by demonstrating the amplification of single-copy DNA template with primer functionalized microbeads in engineered nanoliter droplets (illustrated in Figure 2.2a).[88] Long-range sequencing results generated from ~100 attomol of a 624 bp product demonstrated that these amplicons are compatible with downstream attomole-scale Sanger sequencing. By integrating the technologies presented above, a high-throughput Microbead Integrated DNA Sequencing (MINDS) bioprocessor (Figure 2.2b and 2.2c), coupled with a procedure for whole genome *de novo* sequencing (see details in Box 2.1), can be envisioned. The target performance of this integrated microsystem is ~50,000 bases per hour and 1 Mb/day with read lengths over 600 bases and 30 min processor cycle time. While integrating all these elementary steps into a single device is an engineering challenge, we believe it is only a matter of time before the MINDS bioprocessor is fully realized. This integrated system will most likely never match the throughput of next-generation sequencing techniques, but it will provide an important platform for medium-throughput personal sequencing and it will be uniquely suited to the *de novo* sequencing of large and complex genomes from single templates or single cells thereby enabling an unprecedented characterization of somatic cell variation.

2.5.2 Gene expression analysis

It is now evident that even genetically identical cells with seemingly identical cell histories and environmental conditions can have significant differences in gene expression levels, due largely to the alteration of mRNA production by random fluctuations or complex molecular switches.[49, 89] Additionally, many biological processes, such as stem cell differentiation,[90] and diseases, such as cancer,[91] are triggered by single cell variation. The highly heterogeneous gene expression and microRNA levels in these cell populations are often missed by conventional techniques, which usually analyze (and therefore average) 10^3-10^6 cells. Quantitatively studying gene expression on the single-cell level is imperative to accurately understand the true mechanisms behind cellular processes. Reverse transcription PCR (RT-PCR) is valuable for single-cell gene expression analysis; by translating RT-PCR assays into microfluidic formats, significant improvements can be achieved in cell manipulation, throughput, and sensitivity ultimately to the single cell level.

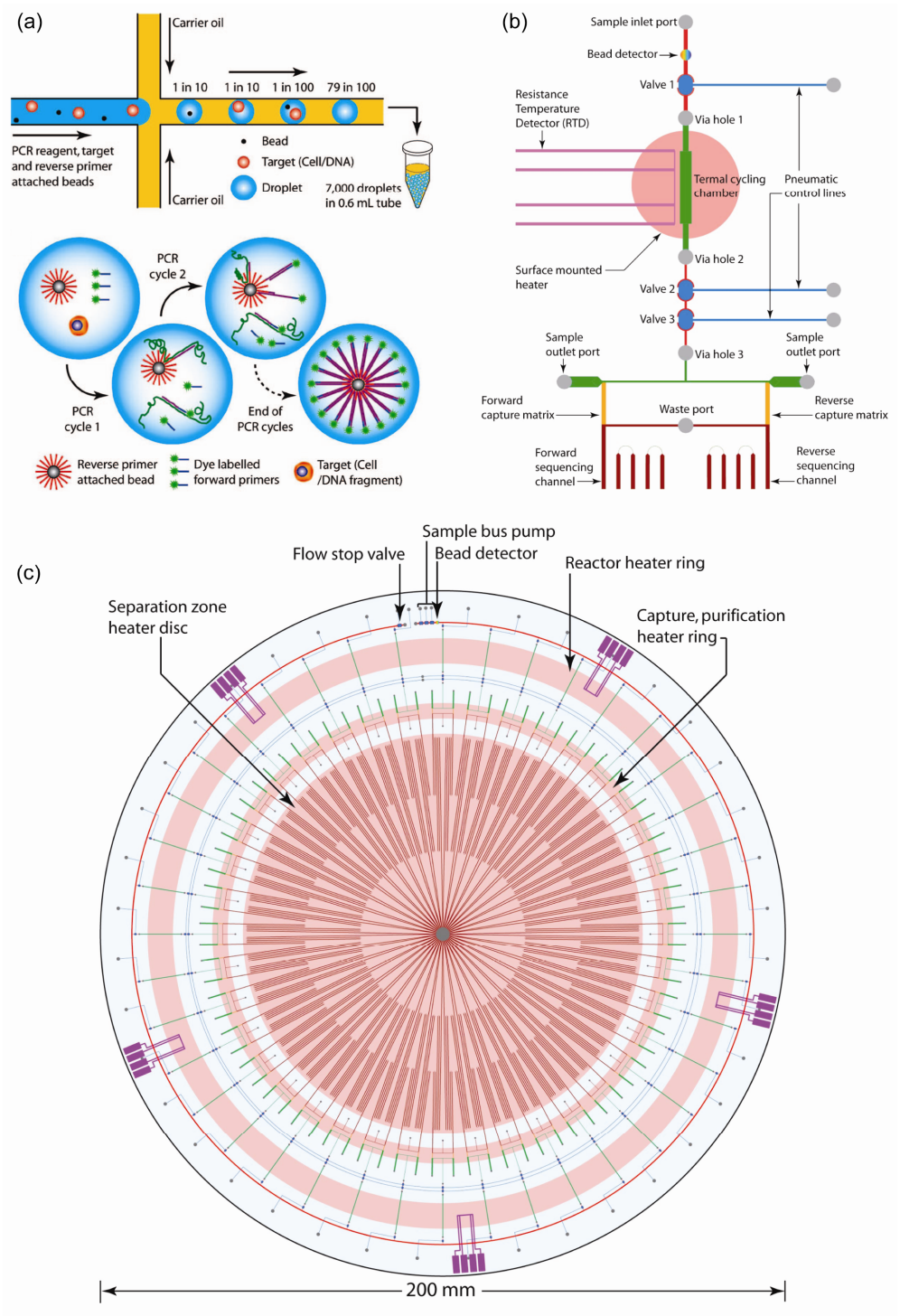

Figure 2.2. Integrated high-throughput nanoliter-scale bioprocessor for 100-attomole DNA sequencing generated from single templates in droplets.

(a) Schematic of single cell or single copy genetic analysis (SCGA) in nanoliter droplets. Beads and template are diluted in PCR solution and pumped through a microfabricated droplet generator to form uniform PCR mix droplets. The number of droplets containing a single template copy and a single bead conforms to the Poisson distribution. Each functional droplet contains a bead modified with reverse primers, fluorescent labeled forward primers in solution, and a single template. Amplification produces a large number of double-stranded DNA products that are

linked to the bead by the reverse primer. After amplification, the beads are isolated and pooled, before being run through a flow cytometer to determine the distribution of fluorescence on each bead and to isolate possible PCR colonies. (Adapted with permission from Reference 88) (b) Schematic design of double-ended sequencing chip. A diluted bead solution obtained from SCGA is pumped into the nL-scale thermal cycling chamber to place only one bead in the reactor. After thermal cycling, the extension fragments (forward and reverse) are pumped in parallel to two separate capture gels, where one lane selectively captures the forward extension fragments and the other the reverse. After capture, the chip is heated to 70 ^{o}C and the dehybridized purified band is injected into the sequencing column for separation. (c) Schematic design of a 40 unit array MINDS processor for high-throughput nanoliter-scale paired end Sanger DNA sequencing on the 200-mm-diameter wafer. The processor consists of 40 thermal cycling reactors, 80 sample purification and concentration chambers and 80 separation channels with a common central anode. A single sample bus channel along with one sample inlet port and one bead detector address all 40 microprocessors.

Box 2.1. The Microbead INtegrated DNA Sequencing (MINDS) method for whole genome *de novo* sequencing.

Genomic DNA is first randomly sheared into ~ 2 Kb fragments and inserted into plasmid DNA using standard methods. After that, millions of nanoliter volume water-in-oil droplets containing individual plasmids and primer functionalized microbeads are generated using a high-throughput microfabricated droplet generator. Thousands of these droplets are collected in a single tube and simultaneously PCR cycled in conventional PCR thermal cyclers. In the next step, the droplets are lyzed and the microbeads are collected and rapidly sorted using a fluorescence activated cell sorting (FACS) machine to separate the clonal beads from the non-clonal ones. The sorted clonal beads are then introduced into the MINDS bioprocessor (shown in Figure 2.2), which integrates Sanger sequencing reaction, purification, inline injection, and capillary electrophoresis on a single device, for high-throughput nanoliter-scale paired end Sanger DNA sequencing. Compared with conventional whole genome Sanger sequencing, the MINDS method can achieve saving of two to three orders of magnitude with regard to cost, time and space by employing a nanoliter-volume integrated process.

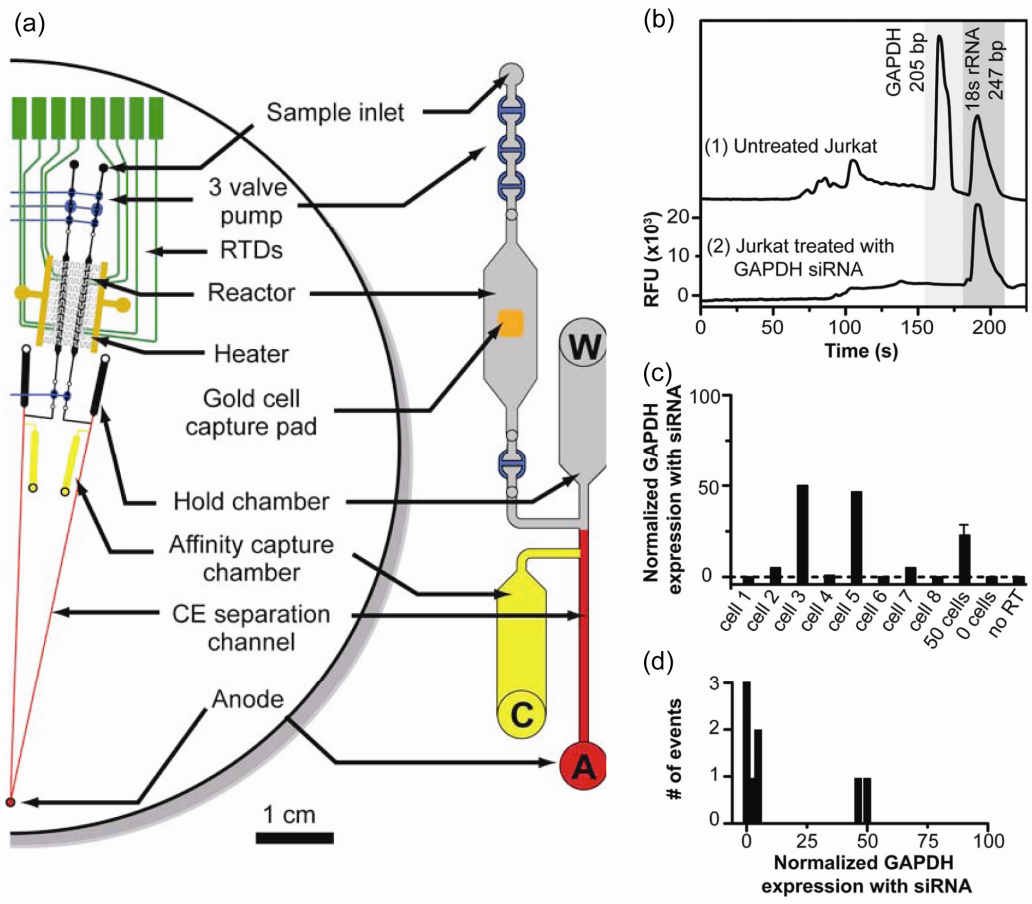

Figure 2.3. An integrated microdevice for gene expression analysis of single cells.

(a) Schematic of the chip layout. This 4-layer PDMS-glass hybrid device has 4 independent systems, each of which contains a 3-valve pump (blue) for sample movement, a size-limited gold pad (gold) for cell capture, a 200-nL reactor with RTDs and a heater for thermal cycling, a hold chamber and an affinity capture chamber (yellow) for post-PCR capture and inline injection, and a CE separation channel (red). (b) Single-cell gene expression and silencing experiments performed on the microdevice. An untreated Jurkat cell shows a 200-bp (GAPDH) and a 247-bp (18S rRNA) peak in the electropherogram, while a cell treated with siRNA shows only a single peak for 18S rRNA. (c) Gene expression of GAPDH from 8 individual Jurkat cells show GAPDH mRNA levels at 0, 5, 50, 1, 48, 0, 5, and 0%. However, GAPDH expression measured from 50 cells shows an average of 21±4 %. (D) Histogram of the number of events for siRNA treated cells shows that there are 2 distinct populations of cells whose expression levels are very distinct from the population average. (Adapted with permission from Reference 92)

For example, Quake's group constructed a multilayer PDMS microsystem that was capable of performing single-cell lysis, followed by affinity mRNA purification and cDNA synthesis.[65] Several chip components integrated into this microsystem assured the success of quantitative on-chip mRNA analysis: accurate fluid control achieved by on-chip microvalves and micropumps, enhanced reaction efficiency provided by nL-scale reactors, and reduced risk of sample degradation due to enclosed structures. Moreover, a key feature of this system is the utilization of oligo(dT)-modified paramagnetic beads for mRNA and cDNA capture, which provide efficient product transport without sample loss and dilution during buffer exchange and product collection. Using similar technologies, Zhong et al. constructed an improved system for extracting total mRNAs and synthesizing cDNA from single human embryonic stem cells.[93] Bontoux and coworkers successfully integrated single-cell trapping, total mRNA extraction, and RT-PCR into a microdevice with rotary reactors and PDMS microvalves.[94] Both systems demonstrated increased efficiency of extraction and reaction due to smaller volumes and integrated analytical processes. However, the dependence on subsequent off-chip amplification and detection, limited their throughput and sensitivity.

Integrated PCR-CE microdevices developed for DNA amplification and separation should be excellent platforms for gene expression analysis using RT-PCR from single cells, due to their excellent sensitivity, integrated operation, and the potential for high throughput. However, until recently no such microsystem was available due to the lack of efficient sample/product transfer between each step. Toward this end, our group recently reported a fully integrated microsystem (Figure 2.3) that is capable of performing single-cell capture, RT-PCR, post-PCR product capture, inline injection and CE separation.[92] The 4-inch-diameter four-lane microdevice has several unique structures that enable quantitative and sensitive analysis: i) a gold pad in a RT-PCR reactor for single-cell capture;[95] ii) a 200-nL reactor for one-step RT-PCR in 25 min;[96] iii) a affinity gel capture structure for post-PCR purification and concentration;[97] and iv) a CE separation channel coupled with the capture structure to achieve inline injection and separation. With an estimated detection sensitivity of < 11 mRNA molecules per reactor, this microsystem established the feasibility of performing single-cell gene expression analysis on an integrated device.

2.5.3 Pathogen/infectious disease detection

Microchip technology can also play an important role in pathogen or infectious disease detection, where point-of-care analysis is highly desired. PCR-based detection technology has found the greatest use for pathogen detection because of its speed (less than an hour), sensitivity (down to a single copy), as well as the capability of detecting minute amounts of targets from a huge nonpathogenic background. Many integrated PCR-CE microfluidic systems have been constructed, and demonstrated the capability of rapid, decentralized detection of various pathogens.[64, 98-100] However, one critical drawback of these systems is that they suffer from low or even no PCR amplification when crude samples containing PCR inhibitors are processed. The integration of sample processing steps, including cell isolation and DNA purification, prior to PCR are required to address this problem.

To achieve a 'sample-in-answer-out' capability of identifying pathogens in complex chemical or biological backgrounds, Landers's group developed an integrated microfluidic genetic analysis system (Figure 2.4a-c), which can perform three major DNA processing steps: DNA extraction, PCR amplification, and electrophoretic separation.[72] The entire analysis timeline, as shown in Figure 2.4d, is less than 24 min, which is about 10 times faster than

conventional methods. The successful analyses of *Bacillus anthracis* (anthrax) in 750 nL of whole blood and of *Bordetella pertussis* in 1 µL of nasal aspirate clearly indicate the possible application of this integrated system for rapid and large-scale screening of disease outbreaks.

To achieve efficient sample transfer in integrated microsystems, magnetic beads are an excellent sample transport vehicle because they can be precisely manipulated using external magnets. Beyor et al. recently developed a cell concentration and isolation microdevice using immunomagnetic beads.[75] *E. coli* cells are driven through a fluidized bead bed magnetically immobilized in microchannels using an integrated on-chip pump. High capture efficiency (70%) and a detection limit of 2 cfu/µL could be obtained. More recently, this cell capture structure was integrated into a PCR-CE microdevice to achieve cell preconcentration, purification, PCR, and capillary electrophoretic analysis on a single device.[101] Since the magnetic beads can efficiently capture and concentrate target cells in the microchannels, and the cell-bead conjugates are precisely transported and located into the PCR chamber using on-chip pumps and external magnets, leading to an impressive sensitivity of 0.2 cfu/µL of *E. coli* O157 cells in a 50 µL input volume. O157 cells could also be selectively detected in a thousand-fold commensal background of *E. coli K12*. This cell capture PCR-CE system represents a significant advancement in the development of rapid, sensitive, and specific lab-on-a-chip devices for pathogen detection that address the macro-to-micro interface challenge.

2.5.4 Forensic short tandem repeat typing

According to the National Institute of Justice (NIJ) 2006 Annual Report (http://www.ojp.usdoj.gov/nij/about/annual-reports.htm), officials estimated a backlog of 350,000 rape and homicide cases pending examination in our nation's state and federal crime labs. In addition, compromised samples, including degraded DNA,[28] low-copy-number DNA,[29] and mixtures,[30] are often encountered in forensic investigations and pose unique challenges to short tandem repeat (STR) typing. To overcome these throughput problems, the separation and typing of STR samples amplified from off-chip PCR have been successfully performed on microfabrication CE array devices, demonstrating the extraordinary speed and throughput.[102-104] However, in order to fully appreciate the benefits provided by microfabrication technology, sample preparation steps, such as PCR and post-PCR cleanup, should ideally be integrated into the CE chips.

Liu et al. developed an integrated PCR-CE microdevice for forensic STR analysis, as well as a portable analysis instrument containing all the electronics and optics for chip operation and four-color fluorescence detection.[105] To explore the concept of on-site forensic human identification, we further developed an improved PCR-CE device, a 9-plex autosomal STR typing system, and a complete typing protocol (Figure 2.5).[106] Real-time DNA analyses at a mock crime scene were carried out in collaboration with the Palm Beach County Sheriff's Office. A mock crime scene was investigated following standard procedures, and three blood stain samples were extracted, amplified and typed at the scene. A DNA profile search against a mock CODIS database with a "convicted offender" sample was successfully conducted within six hours of crime scene arrival. This successful demonstration of on-site STR typing at a crime scene validates the feasibility of real-time forensic human identification.

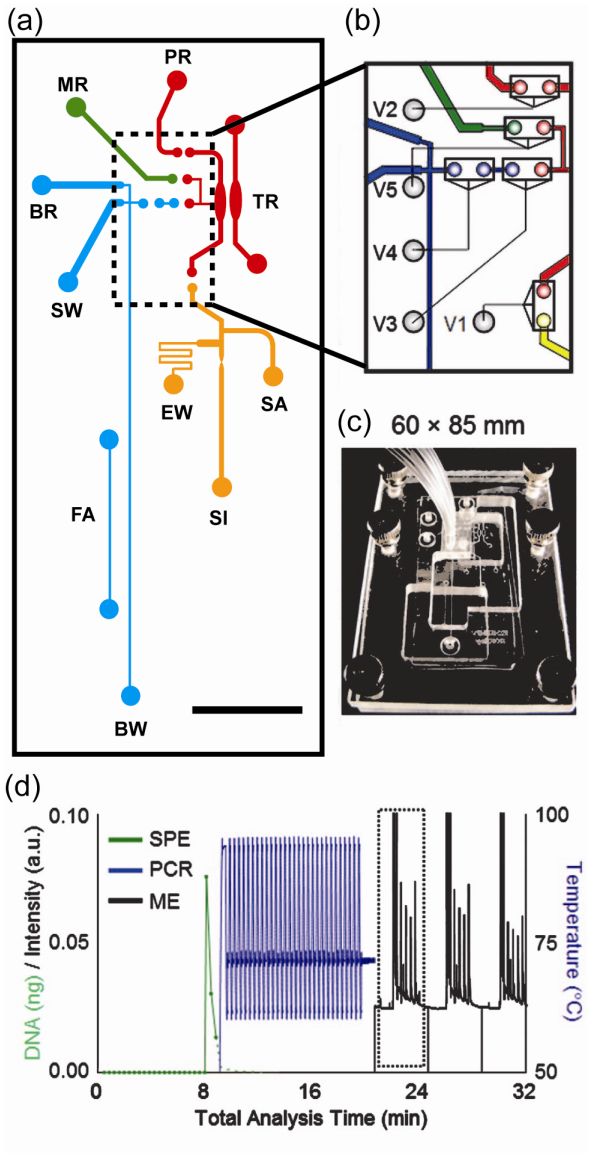

Figure 2.4. A fully integrated microfluidic genetic analysis system for pathogen detection.

(a) A schematic of the microdevice. This device contains three domains for DNA extraction (yellow), PCR amplification (red), and CE separation (blue). All the reservoirs and structures are labeled as: sample inlet (SI), sidearm (SA), and extraction waste (EW) for DNA extraction; PCR reservoir (PR), marker reservoir (MR), sample waste (SW), and temperature reference (TR) chamber for PCR; buffer reservoir (BR), buffer waste (BW), and fluorescence alignment (FA) channel for electrophoresis. (b) Expanded view of the PDMS microvalves integrated on the chip for microfluidic control. (c) A photograph of the chip assembly. (d) The timeline of the entire analysis performed on the microdevice. The green line is the DNA concentration released from the solid-phase extraction (SPE) column as a function of time. The blue line is the temperature cycling profile for PCR. The black line presents the three sequential traces of microchip electrophoresis (ME). (Adapted with permission from Reference 72)

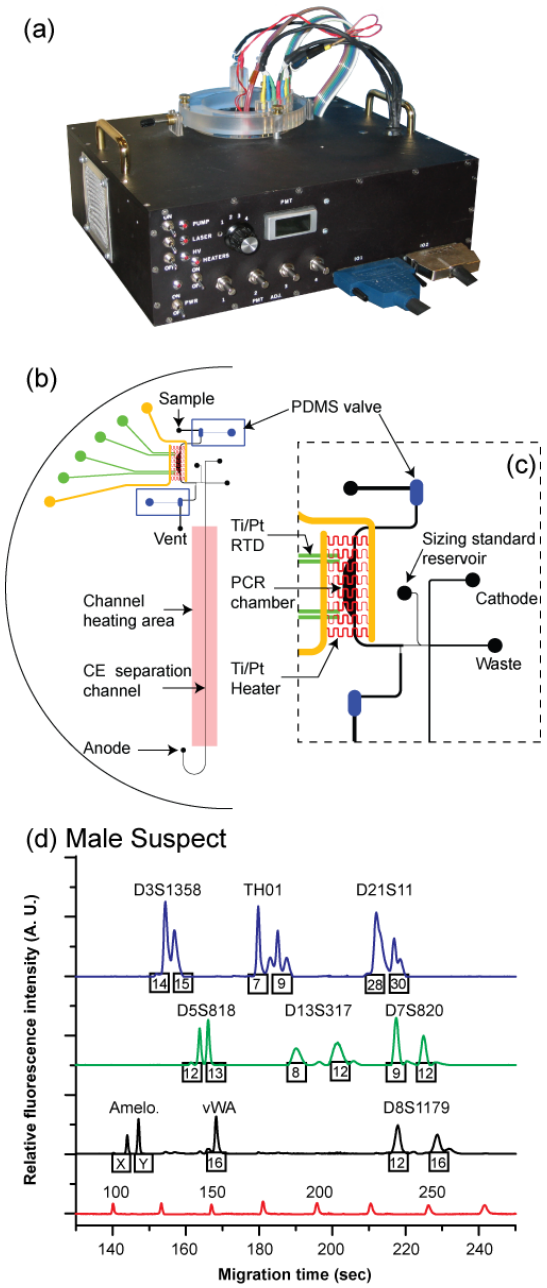

Figure 2.5. A portable integrated genetic analyzer for on-site forensic STR typing.

(a) Photograph of the portable PCR-CE system. (b)Schematic design of the integrated PCR-CE microchip, consisting of a 160-nL PCR chamber with a microfabricated PCR heater and a temperature sensor for on-chip PCR, a co-injector for the injection of a sizing standard, and a 7-cm-long separation channel for electrophoresis. (c) Expanded view of the PCR and injector region. (d) Male suspect's 9-plex STR traces generated on-scene in Palm Beach, Florida using the portable microsystem. This profile was searched against the local CODIS database and a hit was obtained successfully. (Adapted with permission from Reference 106)

In the PCR-CE microdevice discussed above, a traditional cross injector was employed, which produced successful results despite its poor injection efficiency. To address this issue, Yeung et al. developed an integrated device for STR sample cleanup and separation using a method that employs a streptavidin capture gel chemistry coupled to a CE separation channel for forensic STR analysis.[81] Compared to conventional microchip CE with a cross-injector, the fluorescence intensity could be improved 10-50 fold for monoplex samples, and 14-19 fold for 9-plex STR products. This capture structure can be easily incorporated into the high-throughput μCAE and integrated PCR-CE microdevices with the aim to significantly increase the sensitivity, robustness and data quality of low-copy-number and degraded DNA analysis.

2.6 Conclusions and Prospects

Over the past two decades, microfluidic devices for genetic and gene expression analysis have advanced rapidly. Most of the analytical steps have been successfully translated into chip formats where they demonstrated at least 10 times better performance over their conventional counterparts. However, thus far, microfluidic systems are still primarily utilized by the academic research community. We believe fully integrated microfluidic systems, which contain all the necessary analytical components and provide a complete solution to users, will ultimately find wide application, because these integrated systems provide extraordinary advantages which are absent in discrete microdevices with single functions. The reduced reaction volume expedites the assay and enhances the sensitivity; precise fluidic control coupled with efficient sample transport prevents sample loss or dilution and increases the assay sensitivity; and automated operation saves labor and time while eliminating the risks of contamination. While robotics provides macro-scale integration of analytical processes, microfluidics provides micro-scale integration and should be thought of as a microfabricated liquid robot operating at the pL-nL volume scale.

Considering potential needs in the future, several promising “killer apps” are emerging: first, portable genetic analysis instruments are now available with sample-in-answer-out capability for on-site human identification or pathogen detection. This application is timely because biothreats are of increasing concern for military and civilian populations. Second, integrated DNA sequencing systems which can reduce the total cost of sequencing by two orders of magnitude (down to $100,000 genome) will yield great benefits to all genome-related research. Third, microfluidic systems with single-molecule or single-cell sensitivity will enable pioneering research in genetic studies of somatic variation in especially the stem cell and cancer areas.

Acknowledgements

We thank Robert G. Blazej, Palani Kumaresan, and Samantha A. Cronier for providing figures of the MINDS bioprocessor. We also thank Richard Novak, Samantha A. Cronier, Yong Zeng, Amy Twite and Erik C. Jenson for valuable discussions. This work was supported by Grant No. 2007-DN-BX-K142 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice. R.A.M. discloses a financial interest in Microchip Biotechnologies, Inc. (Dublin, CA) who are commercially developing aspects of the technologies presented here.

Chapter 3

Integrated Portable PCR-Capillary Electrophoresis Microsystem for Rapid Forensic Short Tandem Repeat Typing

Reproduced from “Integrated Portable PCR-Capillary Electrophoresis Microsystem for Rapid Forensic Short Tandem Repeat Typing” by Peng Liu, Tae seok Seo, Nathaniel Beyor, Kyoung-Jin Shin, James R. Scherer, and Richard A. Mathies in Analytical Chemistry, 79, 1881-1889 (2007), with permission from the American Chemical Society.

3.1 Summary

A portable forensic genetic analysis system consisting of a microfluidic device for amplification and separation of short tandem repeat (STR) fragments as well as an instrument for chip operation and four-color fluorescence detection has been developed. The microdevice performs polymerase chain reaction (PCR) in a 160-nL chamber and capillary electrophoresis (CE) in a 7-cm-long separation channel. The instrumental design integrates PCR thermal cycling, electrophoretic separation, pneumatic valve fluidic control, and 4-color laser excited fluorescence detection. A quadruplex Y-chromosome STR typing system consisting of amelogenin and three Y STR loci (DYS390, DYS393 and DYS439) was developed and used for validation studies. The multiplex amplification of these four loci with 35 PCR cycles followed by CE separation and 4-color fluorescence detection was completed in 1.5 hr. All the amplicons can be detected with a limit of detection of 20 copies of male standard DNA in the reactor. Real-world forensic analyses of oral swab and human bone extracts from case evidence were also successfully performed. Mixture analysis demonstrated that a balanced profile can be obtained even at a male-to-female template ratio of 1:10. The successful development and operation of this portable PCR-CE system establishes the feasibility of rapid point-of-analysis DNA typing of forensic casework, of mass disaster samples, or of individuals at a security checkpoint.

3.2 Introduction

Short tandem repeat (STR) assays have become an indispensable and routine technique in modern forensic casework since their first application in 1991.[107] Polymerase chain reaction (PCR)-based amplification of multiple STR loci followed by capillary electrophoretic (CE) separation provides STR assays with high sensitivity and high discrimination power.[15, 27, 108] In addition to forensic identification, STR assays have found application in paternity testing, missing person investigations, human identification in mass disasters, evolution and clinical diagnosis.[9, 10] However, the limited capabilities of current genotyping technologies, which are time-consuming, labor-intensive and expensive, have resulted in backlogs in forensic laboratories around the world. To address these issues, high-throughput and integrated instruments are needed to improve the data productivity. In addition, rapid and portable DNA typing devices that can provide on-site forensic analysis could be valuable in crime scene investigation, and for law enforcement and security applications.

In the quest to produce portable, real-time analytical devices as well as high-throughput analyzers, microfabricated microfluidic analysis systems, so-called micro-total analysis systems (μTAS), have attracted increasing attention due to their ability to integrate multiple molecular biology processes at μL-nL scale in a single device. Since the inception of μTAS in 1990,[41] much progress has been made to miniaturize and integrate DNA-analysis steps into a microchip format,[109, 110] including DNA extraction,[111-113] PCR amplification[114, 115] and CE separation.[84, 85, 116] These technologies are now beginning to be translated to forensic applications.

In 1997, Ehrlich's group demonstrated that a quadruplex STR system (*CSF1PO, TPOX, THO1, and vWA*) could be separated with high accuracy in less than 2 min by microchip capillary electrophoresis.[102] More recent work in our group demonstrated the use of a 96-channel microfabricated capillary array electrophoresis (μCAE) device coupled to a 4-color confocal fluorescence scanner for high-performance STR typing using both the PowerPlex 16® and AmpFℓSTR® Profiler Plus® multiplex PCR systems.[103] The separations were completed in less than 30 minutes with single-base resolution on 96 CE channels simultaneously. Although these systems heavily rely upon conventional off-chip sample preparation, they do indicate that chip-based CE technology is poised for application in forensic laboratories.

The on-chip integration of DNA sample preparation by PCR has also been demonstrated. An integrated PCR-CE microdevice consisting of a silicon reaction chamber attached to a glass CE analysis chip was developed in our laboratory in 1996 to amplify and analyze PCR products, providing rapid reaction times, low sample consumption, and potential on-chip integration with other analytical techniques.[79] Since then, great progress has been made in the development of PCR microdevices, including alternative chip formats (flow-through and stationary chamber), substrate materials (silicon, glass and polymer), and heating methods (contact and noncontact heating).[117, 118] However, most of these systems either require a high starting template concentration or are not suitable for integration with CE separation.

Based on the development of integrated PCR-CE microdevices by Lagally et al.,[58, 119, 120] a fully integrated portable PCR-CE microsystem was recently demonstrated for pathogen detection applications. The limit of detection for this system was 2-3 *E. coli* cells and the amplifications required only 20 min.[64] More recently, a nanoliter-scale microdevice has been developed, which integrates the three Sanger sequencing steps: thermal cycling, sample purification, and capillary electrophoresis.[54] Building on this work, a 4-lane integrated PCR-CE

array microdevice was also demonstrated to amplify femtogram amounts of DNA followed by electrophoretic separation in less than 30 min.[73] These advances raise the possibility that these technologies can also be used for forensics whose stringent requirements include high-efficiency and balanced amplification of multiple STR loci, reproducible electrophoretic separation under denaturing conditions, and high-sensitivity four-color fluorescence detection.

Here we present the design and operation of a new PCR-CE microdevice for forensic STR analysis, as well as a new portable analysis instrument, which contains all the electronics and optics for temperature control, microfluidic manipulation, CE separation, and four-color fluorescence detection. To explore the utility of this system for forensic DNA typing, a quadruplex STR system was developed with amelogenin, a sex-typing marker, and three Y chromosome STR loci. As over 89% of violent offences are committed by men,[121] Y-STR assays have a unique value in forensic DNA typing, particularly in sexual assault cases.[10, 17, 18] Due to the lack of recombination, Y-STR assays have also become a popular tool for paternity testing, evolutionary studies, and historical and genealogical research.[10] With this quadruplex Y-STR system, we evaluated the limit of detection of the portable PCR-CE microsystem as well as its ability to analyze forensic casework samples and to detect male DNA in a background of female DNA.

3.3 Experimental Section

3.3.1 Microdevice design

The microdevice contains two identical PCR-CE systems, symmetrically arranged on the 4-inch wafer (Figure 3.1A). The structure of each system is similar to the device developed in our group previously,[64] but the design has been adapted for the portable instrument. Each system consists of a 160-nL PCR chamber, an integrated heater, a four-point resistance temperature detector (RTD), two PDMS (polydimethysiloxane) microvalves and a 7-cm-long CE separation channel. The PCR reactor region with the relative positions of the PCR chamber, heater and RTD is shown in Figure 3.1B.

The microdevice is comprised of a glass manifold, a PDMS membrane, a glass heater/channel wafer, and a glass RTD wafer (Figure 3.1C). The PCR chamber (bottom side of the heater/channel wafer) and the RTD (top side of the RTD wafer) are laid next to each other after bonding. The microfabricated PCR heater is deposited on the top side of the heater/channel wafer and covers the PCR chamber and the RTD to facilitate thermal cycling under the control of the temperature feedback from the RTD. The PCR chamber contains three exits, two of which are connected to a loading reservoir and a vent reservoir, respectively, through microvalves for the sample loading. The last exit is coupled to a CE separation channel through a narrow injection channel. The glass manifold wafer actuates the PDMS microvalves for fluidic control.[122]

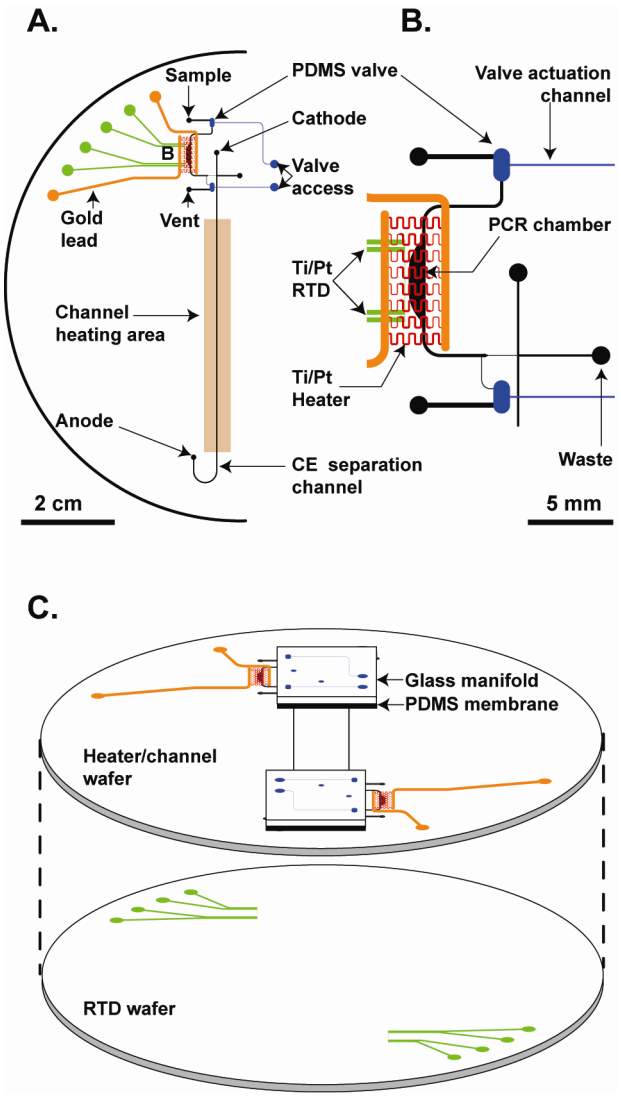

Figure 3.1. The design and structure of the microdevice.

(A) Mask design for the PCR-CE microchip. The glass microchannels are indicated in black, the microfabricated RTD and electrodes are in green, the heater is shown in red, the gold leads of the heater are in gold, and the valves are drawn in blue. (B) Expanded view of the heater, RTD, PCR chamber and CE injector. (C) Exploded view of the assembly of the PCR-CE microchip, showing the RTDs on the upper surface of the RTD wafer, the glass microchannels etched in the lower surface, and the heaters fabricated on the upper surface of the heater/channel wafer.

3.3.2 PCR heater design

The design of the microfabricated PCR heater is intended to create uniform heating over the entire PCR chamber and to facilitate fast thermal response times. In general, the edges of the heater show the most deviation from temperature set point due to the higher thermal dissipation. To adequately maintain the entire chamber volume at a single temperature and keep the thermal mass of the PCR system as low as possible, the thermal power at the extremities of the heater were increased to diminish the temperature deviation. The PCR heater was designed in an iterative process using computational simulation as a guide. As shown in Figure 3.2, an optimized heater design contains 8 serpentine heating elements connected to gold leads in parallel. The width of each heating element in the center part was set to 140 µm. Optimal heating distribution was achieved by narrowing the width to 70 µm on the ends of the central six heating elements, and to 130 µm on the outer two heating elements. Figure 3.2 (Top) shows a color contour plot of the simulated temperature distribution of the PCR chamber at 95 ^{o}C using FEMLAB 2.3 (COMSOL, Inc., Burlington, MA). Using this design method, the temperature differences between the center and the edge of the PCR chamber were reduced to less than 1 ^{o}C in both the X and Y directions. Figure 3.2 (Bottom) presents two typical PCR cycles. The temperature ramp rates can reach 11.5 ^{o}C/s for heating and 4.7 ^{o}C/s for cooling without any active cooling.

3.3.3 Microfabrication

The microfabrication process is similar to that described previously.[58, 64] Briefly, to form the heater/channel wafer, a 550-µm thick D263 glass wafer was coated with 2000-Å amorphous silicon on one side and 200-Å Ti and 2000-Å Pt on the other side. The channel pattern was photolithographically transferred to the amorphous silicon side, and then the sacrificial silicon was etched using SF_6 in a parallel-plate reactive ion etching system creating a hard mask for subsequent glass etching. The exposed glass was etched to a depth of 38 µm in a 49% hydrofluoric acid (HF) bath. After etching, the photoresist and silicon were removed using acetone and SF_6, respectively. The integrated PCR heaters were fabricated on the Ti-Pt side of the same wafer. Using a backside contact aligner, a pattern defining the gold heater leads was photolithographically transferred to the surface. Gold was electroplated onto the open Ti-Pt seed layer to a thickness of 5 µm to form the heater leads. Photoresist was then removed and the wafer was repatterned to define the heating elements. Using an ion beam etching system the heating elements were etched into the Ti-Pt seed layer. Finally, holes were drilled using a CNC mill for via holes, fluidic reservoirs, as well as electrical and pneumatic access holes.

To form the RTD wafer, a 700-µm D263 glass wafer coated with 200-Å Ti and 2000-Å Pt was patterned with photoresist and etched using hot aqua regia. To form the glass microchannels and PCR chambers, the RTD wafer and the heater/channel wafer were thermally bonded in a vacuum furnace at 580 ^{o}C for 6 hr. The glass manifold was fabricated from a 700-µm D263 glass wafer using the same glass etching method described above, and diced into 23 × 18 mm pieces. The microvalves were assembled by cleaning the PDMS membrane in a UV-ozone cleaner for 1 min and then sandwiching the membrane between the bonded wafer stack and the glass manifold. This method results in a tight but reversible glass-PDMS bonding.

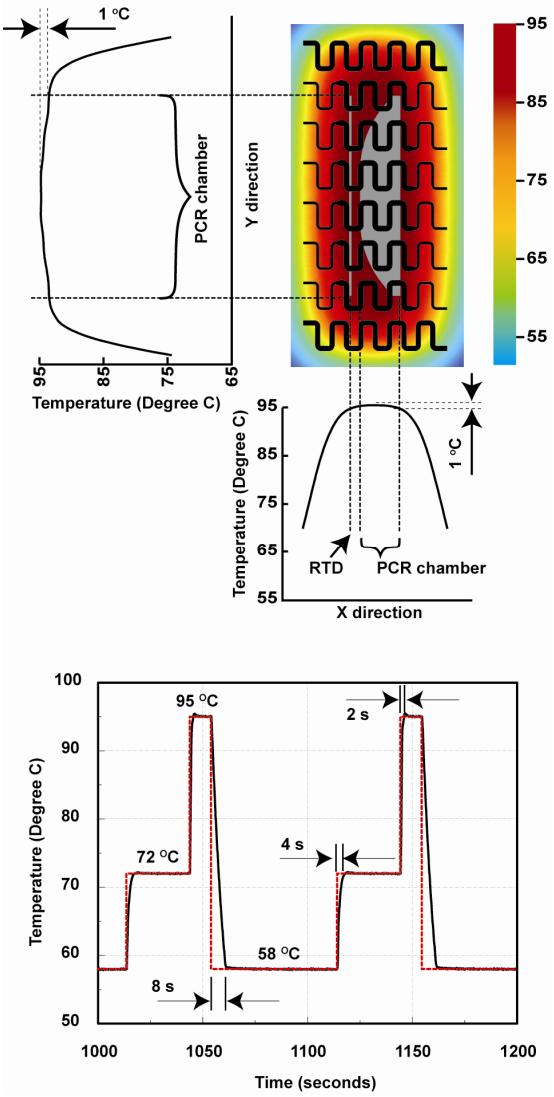

Figure 3.2. The design of the microfabricated PCR heater.

(Top) Color contour plot of the simulated temperature distribution of the PCR chamber layer at 95 ^{o}C. By varying the widths of the heating elements in the different regions of the heater, a uniform profile was achieved. The differences between the center and the edge of the heater are only 1 ^{o}C. (Bottom) Thermal cycling amplification profile. Black line shows the measured temperature from the RTD and red line is the set temperature. Temperature ramp rates were 11.5 ^{o}C /s for heating and 4.7 ^{o}C /s for cooling.

3.3.4 Instrumentation

The instrument used to perform analyses with the microdevice is shown in Figure 3.3A and 3.3B. The instrument contains a 488-nm frequency doubled diode laser, an optical system for detecting four different fluorescence signals, pneumatics for the on-chip PDMS microvalves, electronics for PCR temperature control, and four high voltage power supplies for CE. The weight of the instrument is 10 kg with a power consumption of 20 W, which can be supplied by a car battery. A LabVIEW graphical interface (National Instruments, Austin, TX) developed in-house was used to control the system through two DAQ boards (National Instruments).

The schematic of the detection system is shown in Figure 3.3C. The beam from the laser (Protera, Novalux Corp., Sunnyvale, CA) is reflected by a dichroic mirror (505DCXT, Chroma Technology Corp., Brattleborro, VT) into an attenuator that limits the power intensity of the laser beam to 4 mW (measured from the objective). Then, the attenuated beam is reflected by a second dichroic mirror (505DCXT), passes though a dichroic beamsplitter (488DCSXBP, Chroma), and is focused into the channel in the microdevice with a custom-built objective (0.70-mm focal length in D263 glass, 0.88 NA). The returning fluorescent signal is collected by the objective and reflected into a four-color confocal detection cube by the dichroic beamsplitter. As shown in Figure 3.3D, the detection cube separates fluorescent light into four distinct channels, blue (505-530 nm), green (530–560 nm), yellow (560–595 nm), and red (>595 nm), by sequential reflection from a serial of dichroic beamsplitters (595DCXR, 570DCXR, 537DCLP, Chroma). Fluorescent light is further filtered by a filter in each channel (Ch1: D520/26m, Ch2: D550/20m, Ch3: D580/26m, Ch4: E600LP, Chroma). The filtered light is focused by an achromat lens (45208, Edmund Optics, Barrington, NJ) into an optical fiber (Newport Corp., Irvine, CA), the entry of which functions as a confocal pinhole and is provided with xyz adjustment, and then guided by the optical fiber into PMT (H9306-03, Hamamatsu Corp, Bridgewater, NJ). Together these signals are processed using an active 5-Hz low-pass filter and collected at 10 Hz using the 16-bit DAQ board.

The microdevice is placed onto a recessed area on the top of the instrument and held in place with a plexiglass manifold. Two 1/2'' PDMS spacers are used to support the manifold and provide a soft contact to the microdevice. The manifold contains six spring-loaded pins pressed against the electrical pads on the device, providing the connections for sensing the RTD and powering the PCR heater. The manifold also contains Pt electrodes that are positioned within the reservoirs on the microchip for application of high voltages during CE. A thin-film heater, (9.2 Ω, Minco, Minneapolis, MN), sandwiched between the microchip and a magnet, is used to heat the CE separation channel. Flush contact between the magnetic heater and the chip is obtained by embedding a steel bar on the surface of the instrument.

The design of the electrical circuits for driving the RTD and heater is the same as presented eariler.[64] Briefly, a 4-mA current source powers the RTD through the outer set of leads, and the resulting voltage is sensed through the inner set. The signal is processed using an active low-pass filter at 5 Hz, and then transferred into the DAQ board. Temperature control is accomplished through a proportion/integrator/differentiator (PID) module within the LabVIEW program, which outputs through the DAQ board to control the PCR heater power supply within the instrument from 0 to 10 V.

The PDMS microvalves are controlled using vacuum or pressure supplied through pneumatic connections to the valve access holes on the glass manifold. Eight pneumatic lines are

available for fluidic control. Each line can be switched between vacuum (open valve) and pressure (closed valve) using a solenoid valve (H010E1, Humphrey, Kalamazoo, MI) controlled through the DAQ board. Pressure (4.5 psi) and vacuum (-8 psi) were separately supplied by two rotary pumps (G12/02-8-LC, Thomas, Sheboygan, WI) inside the instrument.

3.3.5 Microdevice preparation

Before operation, the channels were first coated for 1 min with a dynamic coating diluted 1:1 with methanol (DEH-100, The Gel Company, San Francisco, CA) to minimize electroosmotic flow. The separation matrix, 5% (w/v) linear polyacrylamide (LPA) with 6 M urea in 1×Tris TAPS EDTA (TTE) buffer, was loaded from anode reservoir with a syringe to fill the entire CE separation system. A prepared PCR mixture (10 μL) was pipetted into the sample reservoir. Vacuum applied at the vent reservoir moved the sample into the PCR chamber and a gel-solution interface was formed at the end of the narrow injection channel. This interface functioned as a passive barrier to prevent the flow of reagents into the CE channels during thermal cycling. Using this method bubble-free loading of the PCR reactor was consistently achieved. After sample loading, the PDMS microvalves were closed by applying pressure to prevent hydrodynamic flow.

Table 3.1. Locus information, dye labeling and primer sequences

Locus	Repeat Motif	Dye Label	Primer Sequence (5' → 3')	9948 male standard DNA	
				Repeat number	Amplicon Size (bp)
Amelogenin	-	FAM-FAM	CCCTGGGCTCTGTAAAGAA ATCAGAGCTTAAACTGGGAAGCTG	X, Y	106, 112
DYS390	[TCTG] [TCTA]	FAM-R6G	CTGCATTTTGGTACCCCATA GCAATGTGTATACTCAGAAACAAGG	24	171
DYS393	[AGAT]	FAM-TMR	AACTCAAGTCCAAAAAATGAGG GTGGTCTTCTACTTGTGTCAATAC	13	123
DYS439	[GATA]	FAM-ROX	ACATAGGTGGAGACAGATAGATGAT **G**CCTCAAGTGATCCACCCAAC	12	191

The 5' **G** of the DYS439 reverse primer was added to promote adenylation.
FAM: 5-carboxy fluorescein; R6G: Rhodamine 6G; TMR: N, N, N', N'-tetramethyl-6-carboxyrhodamine; ROX: 6-carboxy-x-rhodamine.

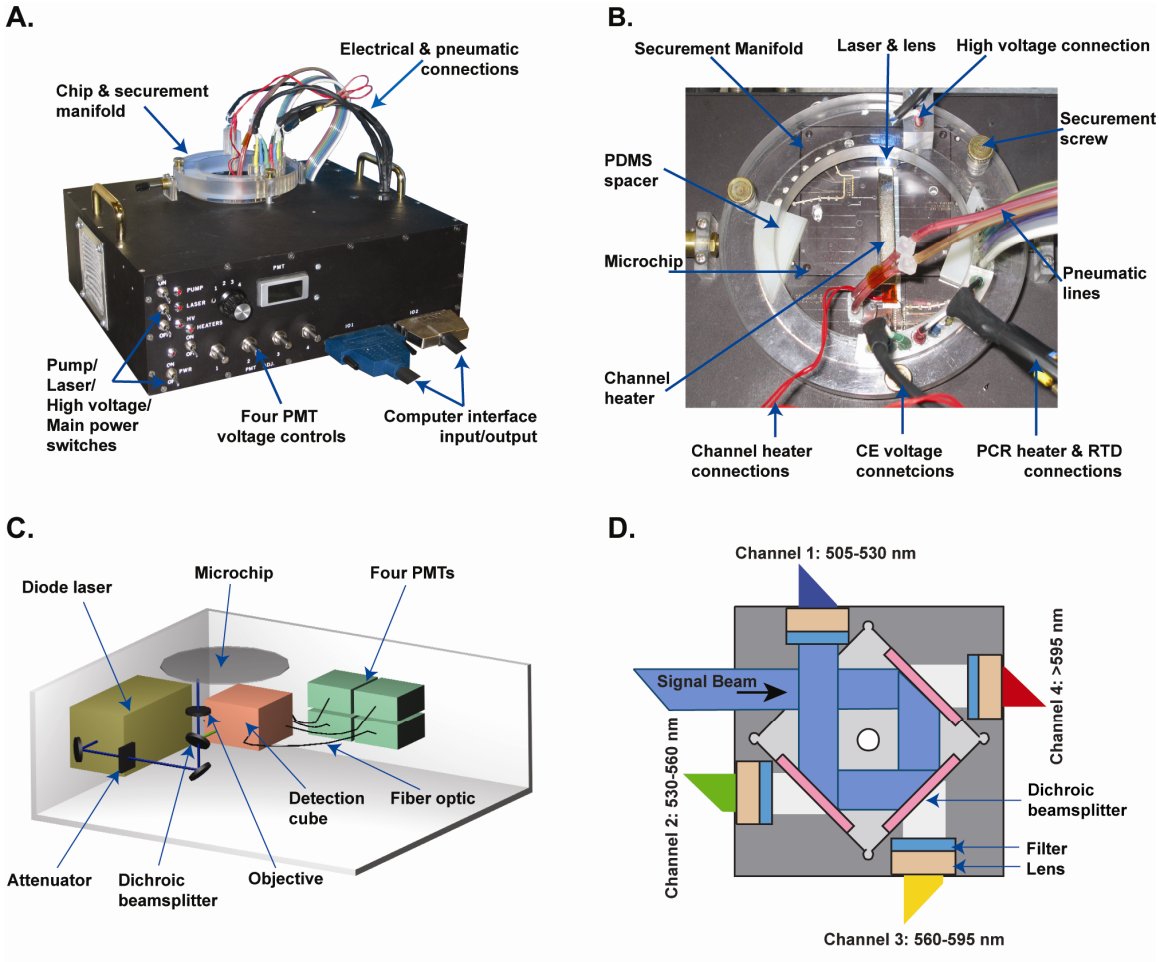

Figure 3.3. The structure of the portable instrument.

(A) Photograph of the portable PCR-CE system. The analysis system box has dimensions 12 × 10 × 4 in. (B) Close-up of the microchip and the manifold. A plexiglass manifold was used to fix the microchip in place and supply the electrical and pneumatic connnections to the chip. (C) The schematic of confocal fluorescence detection system. (D) Expanded top view of the four-color detection cube.

3.3.6 PCR amplification and capillary electrophoresis

PCR amplifications were conducted from 9948 male and 9947A female genomic DNA (Promega commercial genomic DNA controls, Promega, Madison, WI), as well as two samples from forensic caseworks previously processed by the Palm Beach County Sheriff's Office. These casework samples were extracted from an oral swab and human bone, respectively, using the DNA IQ^{TM} system (Promega), and then quantified using Quantiblot (Applied Biosystems, Foster City, CA) with Hitachi CCDBio (Hitachi, Alameda, CA) signal detection. All the DNA templates were also amplified in a thermal cycler and analyzed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) to obtain the sizes of the allele fragments and validate corresponding on-chip results.

The quadruplex STR system included amelogenin and three Y-chromosome STR loci, DYS390, DYS393 and DYS439. Table 3.1 presents the PCR primers and associated dye labels, as well as the expected STR repeat numbers and amplicon lengths. The forward primers were labeled with energy transfer (ET) dye cassettes developed in our group and described previously.[123] The 20-µL PCR mixture prepared for each experiment was comprised of Gold ST*R buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 µg/mL BSA, 200 µM each dNTP) (Promega), templates ranging from 0-50 copies in the 160-nL PCR chamber, primers, and FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). The corresponding primer concentrations in the singleplex PCR amplifications were 150 nM for amelogenin, 80 nM for DYS390, 120 nM for DYS393 and 180 nM for DYS439, respectively. In the multiplex PCR reactions, the primer concentrations were adjusted to 150 nM for amelogenin, 150 nM for DYS390, 120 nM for DYS393 and 180 nM for DYS439. The final DNA polymerase concentration was 0.2 U/µL in all experiments except for the analyses of male DNA in female DNA background, where the concentration was increased to 0.4 U/µL. The thermal cycling protocol was comprised of initial activation of the Taq polymerase at 95 ^{o}C for 4 min, followed by PCR cycle of 95 ^{o}C for 10 s, 58 ^{o}C for 60 s, 72 ^{o}C for 30 s, and a final extension step for 2 min at 72 ^{o}C. For the singleplex reactions 32 cycles were employed while 35 cycles were used for the multiplex.

Following microchip PCR amplification, the CE separation channel was heated to 70 ^{o}C using the channel heater. After the microvalve adjacent to the sample reservoir was opened, the amplified sample was electrophoretically injected into the CE system towards the waste by applying an electric field of ~100 V/cm while floating the anode and cathode. A separation field of 250 V/cm was then applied between the cathode and anode. In the first 5 s of the separation, a backbiasing field of 80 V/cm was applied at the sample and waste, which were floated for the remainder of the separation. Raw electropherograms were processed with BaseFinder 4.0. Processing procedures include baseline adjustment, cross-talk analysis and convolution filtering. After each run, the glass manifold was removed, the PDMS membrane was replaced, and channels and chambers were cleaned using piranha (7:3 H_2SO_4: H_2O_2) to prevent run-to-run carryover contamination.

3.4 Results and Discussion

The quadruplex STR system for testing the portable 4-color PCR-CE microsystem consists of the loci DYS390, DYS393, DYS439 and amelogenin. DYS390, DYS393 and

DYS439 are members of the extended minimal haplotype loci, which play central roles in the current Y-STR DNA typing.[16] The haplotype diversity of these three loci is 0.9473 in the US population. In addition to these three Y-STR loci, amelogenin, which codes for a protein found in tooth enamel, was employed. PCR amplification of this marker produces a 106-bp and a 112-bp amplicon from the X and Y chromosome, respectively. Amelogenin is widely used for sex-typing and sample quality evaluation in the forensic community.[124] In our system, amelogenin serves as a positive control, providing important information about sample quality and amplification performance.

3.4.1 Singleplex and multiplex STR amplification.

Singleplex amplifications on each locus were performed first to examine the functionality of the PCR-CE microsystem as well as the amplification performance of these DNA markers. In these PCR experiments, each DNA marker was amplified from 20 copies of 9948 male standard genomic DNA templates in the 160-nL PCR chamber with 32 PCR cycles. After thermal cycling, the PCR product was immediately injected and separated on the electrophoresis channel. An entire analysis was completed in 1.5 hr. Panel A in Figure 3.4 presents an amplification of the amelogenin marker. A 106-bp X-chromosome and a 112-bp Y-chromosome amplicon labeled with FAM-FAM were observed, indicating that the template is male DNA as expected. Panel B presents an amplification and detection of the DYS390 locus, revealing a 171-bp amplicon labeled with FAM-R6G. Similarly, a 123-bp DYS393 amplicon labeled with FAM-TMR and a 191-bp DYS439 amplicon labeled with FAM-ROX were obtained, respectively, in Panel C and D. With optimized primer concentrations (150 nM amelogenin, 80 nM DYS390, 120 nM DYS393 and 180 nM DYS439), each DNA marker demonstrated a similar amplification efficiency and good sensitivity.

Following successful amplifications on each locus individually, a multiplex PCR-CE experiment was carried out on this four-locus multiplex system. Starting template (50 copies of 9948 male standard genomic DNA) was loaded in the PCR chamber and 35 PCR cycles were performed. Primer concentrations used in the multiplex system were adjusted slightly to maintain balanced peak intensities for each locus (150 nM for amelogenin, 150 nM for DYS390, 120 nM for DYS393 and 180 nM for DYS439). As shown in Figure 3.4 (Panel E), all the peaks (106, 112, 123, 171 and 191 bp) were fully resolved and balanced. Compared with singleplex amplifications, multiplex STR amplifications exhibit lower amplicon yields due to competition between each locus. Therefore, both the initial template copy number (50 copies) and the PCR cycle number (35 cycles) were increased to compensate for this effect.

A limit-of-detection (LOD) analysis for multiplex amplifications of 9948 male standard genomic DNA was performed. Figure 3.5 presents results from a series of amplifications conducted from 0, 20, 30 and 50 copies of template in the PCR chamber with 35 PCR cycles. Even with only 20 copies of DNA template, the multiplex amplification still shows all the expected peaks in the electrophoregram. An amplification from 10 copies was also performed, however, a complete profile was not obtained. The amplicon peak intensities are reduced and show more variability as the initial templates decrease from 50 to 20 copies. When the template copy numbers fall into the low copy number (LCN) amplification range (<100 pg or <33 copies),[27] stochastic effects occur, and repeated amplifications of identical solutions exhibit fluctuations in peak intensity. Finally, it should be noted that the absence of any amplicons in the negative control (0 initial copies) demonstrates the effectiveness of the piranha cleaning conducted after each run.

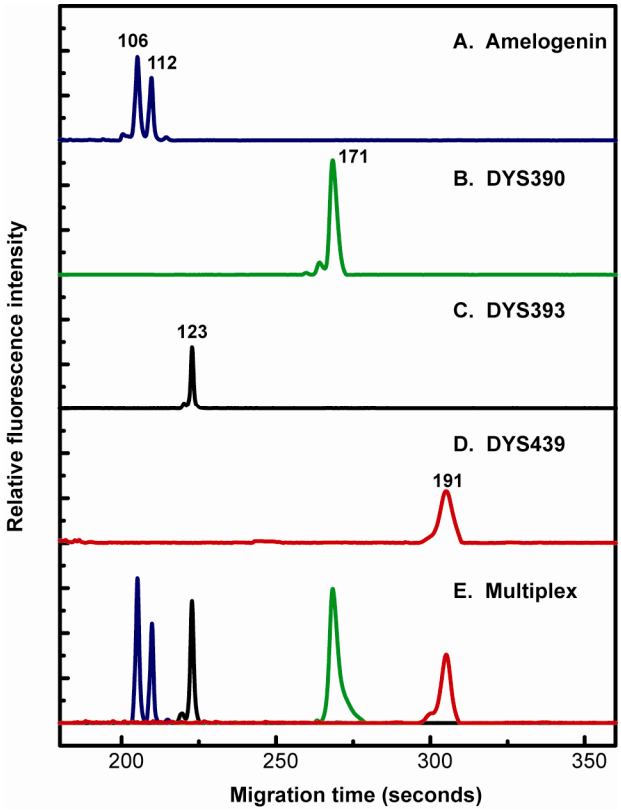

Figure 3.4. Singleplex and multiplex STR amplification performed on the PCR-CE microsystem.

(A) Amplification of the amelogenin marker from male standard genomic DNA. A 106-bp X-chromosome and a 112-bp Y-chromosome amplicon labeled with FAM were amplified from 20 copies of the template with 32 PCR cycles. (B) DYS390 Y-chromosome amplicon (171 bp) labeled with FAM-R6G from standard male genomic DNA. (C) A 123-bp DYS393 amplicon from male standard genomic DNA labeled with FAM-TMR. (D) A 191-bp DYS439 amplicon labeled with FAM-ROX. (E) Multiplex PCR of all four loci from 50 template copies with 35 PCR cycles.

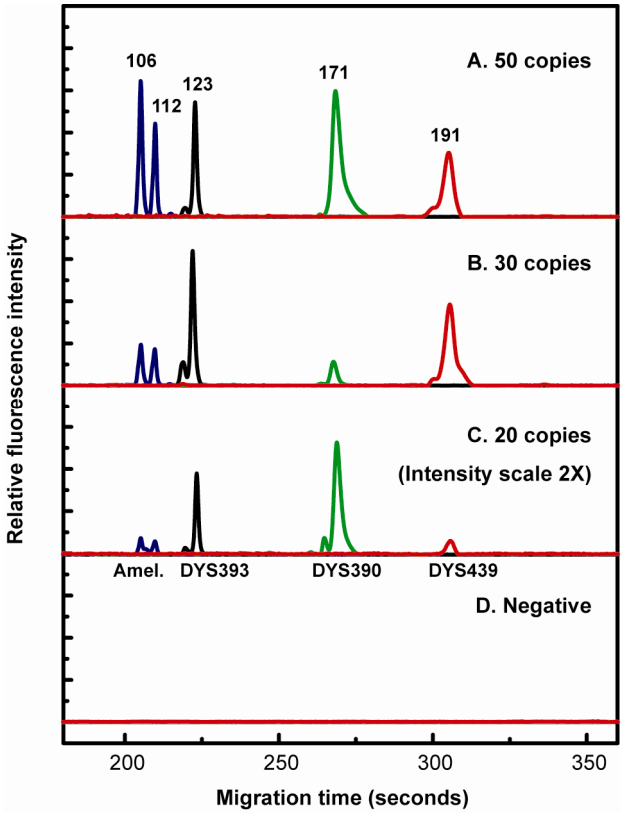

Figure 3.5. Limit of detection for multiplex analyses of 9948 male standard genomic DNA.

PCR cycles were 35 in each case. The trace obtained from 20-copy template was enlarged twice for display. A negative control experiment was performed to confirm the absence of carryover.

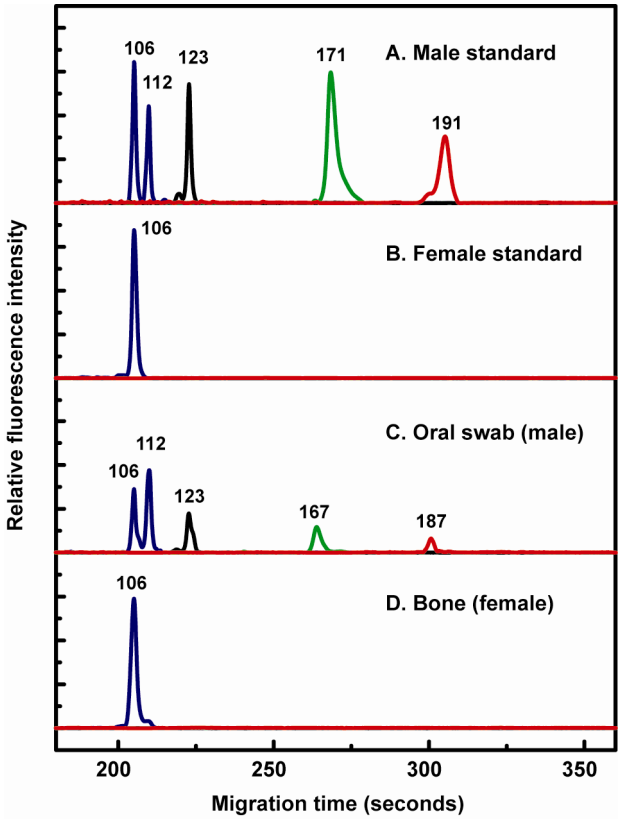

Figure 3.6. Multiplex STR forensic analysis using the PCR-CE microdevice.

These samples are from standard genomic DNA and from samples extracted from an oral swab and human bone, respectively. In each case, 50 starting template copies and 35 PCR cycles were employed. (A) Analysis conducted from 9948 male standard DNA, showing the presence of all the amplicons on these four loci. (B) Analysis of 9947A female standard DNA, showing only the expected presence of the 106-bp X-chromosome peak. (C) Analysis conducted on genomic DNA extracted from an oral swab. All the expected peaks were observed, showing the source is male. (D) Analysis of a human bone sample. Only one 106-bp amplicon was detected, showing the source is female.

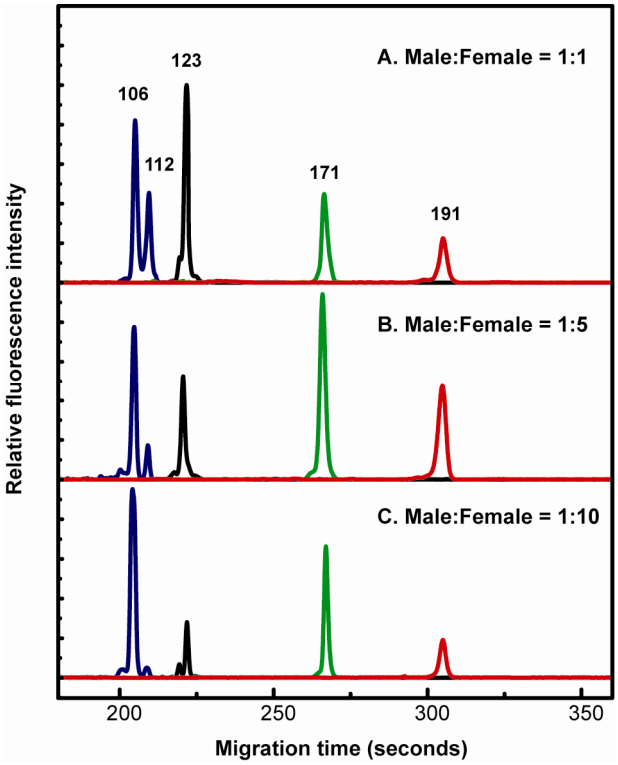

Figure 3.7. Mixture sample analysis using the PCR-CE microdevice.

Multiplex STR analysis of male genomic DNA (50 copies) in presence of a female genomic DNA background using the PCR-CE microsystem (35 PCR cycles). The template ratios of male-to-female range from 1:1 to 1:10. As the ratio increased, the 106-bp amelogenin amplicon from the X chromosome became more and more dominant over the 112-bp Y chromosome product.

3.4.2 Analysis of forensic casework.

Samples obtained from forensic casework usually have lower amplification efficiency, due to PCR inhibitors, which remain with the DNA throughout the sample preparation process,[125, 126] or due to DNA degradation by exposure to environmental elements or natural contaminants.[127] Here we selected two typical samples, one from an oral swab and the other from human bone, which were previously processed and analyzed by the Palm Beach County Sheriff's Office. Buccal cell collection with a cotton oral swab is often used in cases, where reference samples from suspects or family members are needed to perform comparative DNA testing.[128] Human bone remains in forensic caseworks represent one of the most degraded biological materials for PCR-based DNA typing, since they are usually collected after a long period of exposure in a harsh environment, such as burial in soil.[129] Therefore, these two typical samples were chosen to test our integrated PCR-CE forensic system.

Four separate amplifications, including 9948 male and 9947A female standard genomic DNA, which serve as controls, and two casework samples from an oral swab and human bone, were conducted from 50 template copies with 35 PCR cycles. Figure 3.6A and 3.6B present the PCR analyses conducted from male and female standard DNA, showing all the expected peaks with correct gender discrimination. Figure 3.6C presents an amplification and analysis of the DNA sample extracted from an oral swab. All the amplicons in four loci were successfully obtained, indicating the sample is male DNA. Figure 3.6D shows only one peak at 106 bp, corresponding to the successful amplification of female human bone DNA. Off-chip results from ABI Prism 3100 confirmed the genders of these two samples and indicated that the amplicon lengths of the oral swab sample in DYS390 and DYS439 are 167 bp and 187 bp, one repeat less than those corresponding amplicons from 9948 standard DNA. These differences were also observed in the on-chip results, by aligning the profiles of male standard DNA and oral swab sample.

3.4.3 Mixture analysis.

The ability of our system to provide interpretable DNA amplification profiles, when a minute amount of male DNA is present in a high background of female DNA, is very critical, as this situation is often encountered in Y-STR forensic analysis.[10] Quadruplex amplification and detection was carried out by mixing male and female standard genomic DNA together during the sample preparation. The male DNA in each run was maintained at 50 copies, while the female DNA was increased to achieve ratios of male-to-female genomic DNA of 1:1, 1:5 and 1:10, respectively, resulting in ratios of Y-to-X chromosomes of 1:3, 1:11 and 1:21. Since a high yield of the 106-bp X-chromosome product is expected to overwhelm the other Y-chromosome amplicons, the DNA polymerase concentration was increased from 0.2 U/μL to 0.4 U/μL to assure full amplification and to produce balanced profiles. The results of this experiment in Figure 3.7 show that, as the ratio increased, the 106-bp amplicon from X chromosome became more and more dominant over the 112-bp Y-chromosome product. The peak area ratios are roughly equal to the initial template ratios of Y-to-X chromosomes (1:3, 1:11 and 1:21). The other three Y-chromosome loci (DYS390, DYS393 and DYS439) were still fully amplified and balanced in each case. However, slight signal reductions were observed, due largely to the increase of the 106-bp X-chromosome amplicon which used up most of the PCR resources. These data indicate that the system is capable of analyzing male DNA in the presence of a high female DNA background. Although the ratio could be lowered further in amplifications without

the amelogenin marker, additional valuable information, such as the male-to-female DNA ratio, is obtained with this quadruplex system by calculating the peak area ratio of the two peaks in amelogenin.

3.5 Conclusions

A fully integrated PCR-CE microdevice has been optimized for forensic analysis and combined with a new portable instrument including controls for chip operation and 4-color fluorescence detection. This system was used to perform a quadruplex STR forensic analysis; the entire assay was finished in 1.5 hr due to the rapid low-volume (160 nL) thermal cycling and integrated high-speed electrophoretic separation. The detection limit of this system for multiplex amplification of genomic DNA is as low as 20 copies in the PCR chamber. Two real-world forensic casework samples extracted from an oral swab and human bone, respectively, were successfully analyzed, showing the practical application of this system. Finally, male genomic DNA was tested in the presence of excess female genomic DNA background. Intense balanced peaks were observed even at the male-to-female DNA ratio of 1:10.

This microdevice presents a first and significant step towards a fully integrated and portable system allowing highly sensitive, rapid STR analyses in a setting outside a forensic laboratory. For practical forensic applications in the future, a co-injection structure can be included in the microdevice to facilitate running sizing and allelic ladders,[26] and more STR loci should be included to improve the discrimination power. Additionally, autosomal STR typing is under investigation to extend the application range of the portable microsystem. The integrated, high-speed and low-volume STR typing methods developed here will accelerate the forensic identification process and lower the assay cost, thereby reducing backlogs and advancing forensic DNA applications. Furthermore, our demonstration of successful STR analyses on a portable PCR-CE system validates the concept of point-of-analysis DNA typing in crime scene, mass disaster or security checkpoint applications, where rapid on-site human identification is demanded.[9, 130, 131]

Acknowledgement

We thank the UC Berkeley Chemistry Machine Shop for construction of the portable analysis instrument, and Henry Chan of the UC Berkeley Chemistry Electronic Shop for design and fabrication of the electronics. PCR-CE chip microfabrication was conducted at the University of California, Berkeley Microfabrication Laboratory. We thank Amy McGuckian and Cecelia Crouse at the Palm Beach County Sheriff's Office for providing the forensic casework samples and Susan Greenspoon at the Virginia Department of Forensic Science for valuable discussions. We also thank John M. Butler at the National Institute of Standards and Technology for providing the US population data on the three Y-STR loci. This project was supported by Grant No. 2004-DN-BX-K216 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice.

Chapter 4

Real Time Forensic DNA Analysis at a Crime Scene Using a Portable Microchip Analyzer

Reproduced from "Real Time Forensic DNA Analysis at a Crime Scene Using a Portable Microchip Analyzer" by Peng Liu, Stephanie H. I. Yeung, Karin A. Crenshaw, Cecelia A. Crouse, James R. Scherer, and Richard A. Mathies in Forensic Science International: Genetics, 2, 301-309 (2008), with permission from Elsevier.

4.1 Summary

An integrated lab-on-a-chip system has been developed and successfully utilized for real-time forensic short tandem repeat (STR) analysis. The microdevice comprises a 160-nL polymerase chain reaction reactor with an on-chip heater and a temperature sensor for thermal cycling, microvalves for fluidic manipulation, a co-injector for sizing standard injection, and a 7-cm long separation channel for capillary electrophoretic analysis. A 9-plex autosomal STR typing system consisting of amelogenin and eight CODIS core STR loci has been constructed and optimized for this real-time human identification study. Reproducible STR profiles of control DNA samples are obtained in 2 hours and 30 minutes with $\leq$ 0.8 bp allele typing accuracy. The minimal amount of DNA required for a complete DNA profile is 100 copies. To critically evaluate the capabilities of our portable microsystem as well as its compatibility with crime scene investigation processes, real-time STR analyses were carried out at a mock crime scene prepared by the Palm Beach County Sheriff's Office. Blood stain sample collection, DNA extraction, and STR analyses on the portable microsystem were conducted in the field, and a successful "mock" CODIS hit was generated on the suspect's sample within six hours. This demonstration of on-site STR analysis establishes the feasibility of real-time DNA typing to identify the contributor of probative biological evidence at a crime scene and for real-time human identification.

4.2 Introduction

Short tandem repeat (STR) analysis is widely employed as a powerful and indispensable technique for forensic human identification due to its ability to produce highly distinctive profiles from minute amounts of DNA.[9, 27, 108, 132] However, current STR genotyping processes and instruments are labor-intensive, demanding, and one must wait one or more days for results. Accelerating the instrument dependent processes in casework analysis will ultimately assist in reducing DNA backlogs. Micro-total analysis or lab-on-a-chip systems offer tremendous potential for miniaturizing and integrating the steps in forensic STR analysis because it enables the production of dense microfluidic circuits for automated low volume operation.[41, 109, 133, 134] This microtechnology has enabled high-throughput instruments to improve the current STR analysis process, and it should also lead to integrated devices capable of performing on-site forensic DNA analysis to extend the applications of STR analysis.[133-135]

Much progress has been made to integrate the steps of STR analysis, including DNA extraction,[113] PCR amplification[58, 105, 136] and STR fragment separation,[103, 104, 137] into a high-throughput microchip format.[123, 133] Our group demonstrated the use of a 96-channel microfabricated capillary array electrophoresis (μCAE) device for high-performance STR typing using the commercial PowerPlex 16® and AmpFℓSTR® Profiler Plus® kits.[103] The separations were completed in less than 30 minutes with single-base resolution on 96 μCE channels simultaneously. This system has also been successfully evaluated at the Virginia Department of Forensic Science for routine forensic STR analyses.[104] Ehrlich's group also reported the development of a microchip, which can perform separations of 16 purified STR reactions simultaneously in 40 min.[137] Although these microfabricated capillary electrophoresis (CE) devices still rely on conventional off-chip sample preparations, they do demonstrate the speed and throughput capabilities of miniaturized separation systems.

The integration of other sample preparation steps, such as PCR and sample purification, that are necessary for STR analyses, has also advanced. This integration concept has been extensively explored for DNA sequencing,[54] pathogen detection,[64, 71] clinical diagnostics,[53, 138] and gene expression studies[73, 96, 139] on single- or multi-channel formats.[58, 73, 96, 139-141] Integrated forensic DNA sample processing is more challenging due to the stringent requirements for high-efficiency multiplex amplification of STR loci and reproducible electrophoretic separation under denaturing conditions with single-base resolution. Recently, our group developed an integrated PCR-CE microdevice for forensic STR analysis, as well as a portable analysis instrument containing all the electronics and optics for chip operation and four-color fluorescence detection.[105] A variety of control and non-probative casework and mixture sample assays were performed successfully using a simple quadruplex mini-Y STR system, validating the feasibility of point-of-analysis DNA typing.

In the present work, we further explore the concept of point-of-analysis forensic human identification by developing an improved integrated PCR-CE microdevice and typing method capable of conducting real-time forensic STR analysis, and by using this system at a mock crime scene. To enhance the discrimination power, a 9-plex autosomal STR typing system is constructed with amelogenin, a sex-typing marker, and eight CODIS core STR loci. Prior to the field trial, the entire analytical process was evaluated to optimize the amplification efficiency and separation resolution as well as the sizing calibration accuracy using commercial genomic DNA controls. Real-time DNA analyses at a mock crime scene are carried out in collaboration with

the Palm Beach County Sheriff's Office (PBSO). Blood stain collection, DNA extraction, STR analysis on the PCR-CE microdevice, and a DNA profile search against a mock CODIS database with a "convicted offender" sample are successfully conducted within six hours of crime scene arrival. These results establish the feasibility of using our portable PCR-CE microdevice for real-time forensic identification at a crime scene or a security location.

4.3 Materials and methods

4.3.1 Microdevice and instrument

The design of the four-layer glass-PDMS (polydimethysiloxane) PCR-CE microdevice has been modified from that previously developed in our group.[105] The PCR-CE microchip (Figure 4.1) includes a 160-nL PCR reactor with a heater and a four-point resistance temperature detector (RTD) for thermal cycling, two PDMS microvalves for microfluidic control,[122] and a 7-cm-long CE separation channel. In addition, a new coinjector and sizing standard reservoir are integrated into the PCR-CE system to facilitate co-injection of a sizing ladder for STR size calibration.

The microfabrication process follows the methods described previously.[73, 96, 105] The RTDs are photolithographically fabricated on the top of the RTD wafer. All the channel features are isotropically etched to a depth of 38 μm on the bottom side of the heater/channel wafer with hydrofluoric acid. PCR heaters are microfabricated on the top side of the heater/channel wafer. After all the access holes are diamond-drilled with a CNC (Computer Numerical Control) mill, the heater/channel wafer is thermally bonded with the RTD wafer in a vacuum furnace at 580 ^{o}C for 6 hours. The glass manifold with etched channels was diced into 12 × 6 mm pieces. The microvalves are assembled by sandwiching the membrane between the bonded wafer stack and the glass manifold.

The portable genetic analyzer instrument has previously been described in detail.[105] The instrument contains a 488-nm frequency doubled diode laser, a confocal fluorescence optical system for detecting four different fluorescence signals, pneumatics for the on-chip PDMS microvalves, electronics for PCR temperature control, and four high voltage power supplies for CE. A LabVIEW graphical interface (National Instruments, Austin, TX) was used to control the system through two DAQ (Data Acquisition) boards installed in a laptop.

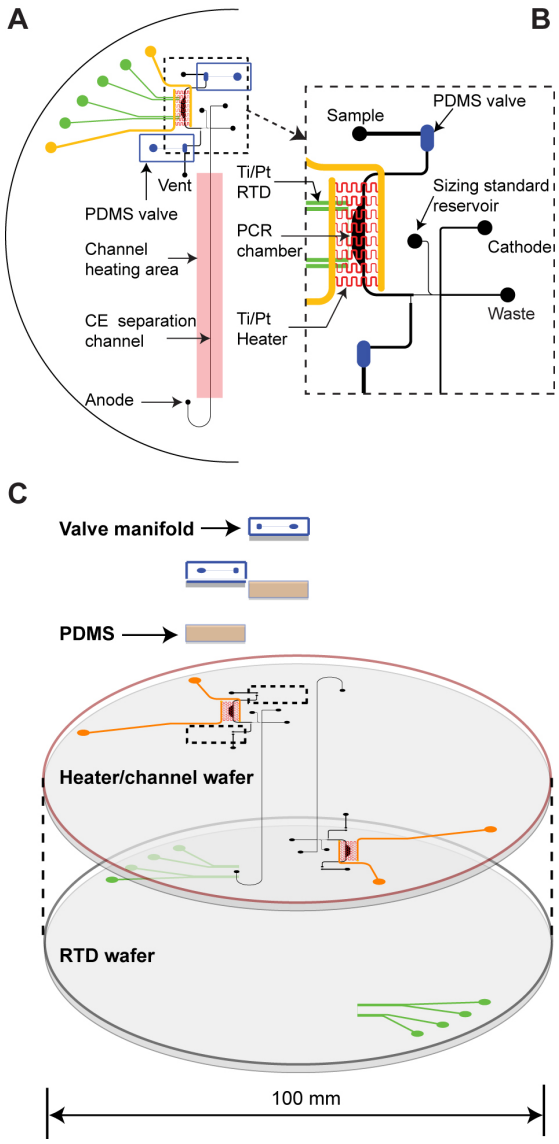

Figure 4.1. The design of the microdevice.

(A) Design of the PCR-CE microchip for forensic DNA analysis. The integrated device consists of 7-cm-long electrophoretic separation channels (black), 160-nL PCR chambers (black), RTDs (green), PCR heaters (red), and PDMS microvalves (blue). A coinjector, including a co-injection channel and a sizing standard reservoir is incorporated into the microdevice. (B) Expanded view of the heater, RTD, PCR chamber and CE co-injector. (C) Exploded view of the PCR-CE microchip assembly showing the valve manifold that controls the PDMS membrane valves, the PCR heaters fabricated on the upper surface of the heater/channel wafer, the glass microchannels etched in the lower surface, and the RTDs on the top of the lower wafer.

4.3.2 PCR amplification and capillary electrophoresis

Two standard commercial genomic DNA controls, 9947A female and 9948 male DNA (Promega, Madison, WI), were employed in the standard DNA test and limit-of-detection analysis. A 9-plex autosomal STR typing system is constructed using primer sequences employed in PowerPlex® 16 (Promega).[37] It consists of the sex-typing marker, amelogenin, and eight STR loci (D3S1358, THO1, D21S11, D5S818, D13S317, D7S820, vWA, and D8S1179) used by the FBI Combined DNA Index System (CODIS) with a size range of 106–258 bp. A 10× primer mixture is prepared by combining primer pairs for each locus (Integrated DNA Technologies, Coralville, IA) according to the primer concentration protocol developed from the PowerPlex® 16 typing system. The 10-µL PCR mixture prepared for each experiment is comprised of 1.5× Gold ST*R buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 µg/mL BSA, 200 µM each dNTP) (Promega), DNA templates at the empirically dervived amount, the primer mixture, 6 U of FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN), and deionized water.

The microchip is prepared for PCR-CE analysis following the same protocol as described previously.[105] The microchannels are coated with a 50% dynamic coating (DEH-100, The Gel Company, San Francisco, CA) diluted in methanol for 1 min to minimize electro-osmotic flow. The separation matrix, 5% (w/v) linear polyacrylamide (LPA) with 6 M urea in 1×Tris TAPS EDTA (TTE) buffer, is loaded into the CE separation channel and the PCR cocktail is pumped in the PCR reactor. The modified thermal cycling protocol starts with an initial activation of the Taq polymerase at 95 ^{o}C for 4 min followed by an initial template denaturing at 96 ^{o}C for 1 min. In the first 10 of 32 PCR cycles, the temperature is held at 94 ^{o}C for 30 s denaturing, then ramped to 60 ^{o}C at 0.5 ^{o}C /s for 30 s annealing, and then to 70 ^{o}C at 0.2 ^{o}C /s for 30 s extension. In the next 22 cycles, all the PCR parameters are kept the same except for the denaturing temperature which is reduced to 90 ^{o}C. Finally, a post extension step is performed at 70 ^{o}C for 10 min. Total PCR time is about 2 hr.

Once the thermal cycling is complete, the denaturing CE separation is performed on a channel preheated to 70 ^{o}C. The MegaBACE™ ET550-R sizing standard solution (15 µL, GE Healthcare, Piscataway, NJ) is pipetted into the sizing standard reservoir. The microvalve adjacent to the sample reservoir is opened, and the amplified sample and the sizing standard are electrophoretically injected towards the waste reservoir simultaneously by applying an electric field of ~100 V/cm on the PCR reactor channel and co-injection channel while floating the anode and cathode. A separation field of 250 V/cm is then applied between the cathode and anode for electrophoresis. At least three injections and separations are performed to confirm the results, and each separation only takes about 8 min. After each analysis, the glass manifolds are removed, the PDMS membrane is replaced, and channels and chambers are cleaned using a fresh piranha solution (7:3 H_2SO_4: H_2O_2) to prevent carryover between runs.

4.3.3 Data acquisition and analysis

The four-color fluorescence data are first converted to binary format and appended with proper header information by a custom LabVIEW program. The preprocessed data files are then analyzed for allele calling using the MegaBACE™ Fragment Profiler 1.2 (GE Healthcare) program which performs baseline and color cross-talk correction. Each allele was designated using the "bins" generated from the allelic ladder in the MegaBACE™ program. In the field test, the electropherograms were analyzed and recorded under the review of a second analyst. The

STR profile of the male suspect was input into a computer and sent to PBSO by email for a CODIS search. The search result was sent back to the scene by phone within 20 min.

4.3.4 DNA extraction, quantification, amplification and DNA profile verification

Four commercial DNA extraction methods were evaluated: DNA-IQ™ (Promega), QIAamp™ (Qiagen, Valencia, CA), Ultraclean™ (MO BIO, Carlsbad, CA), and DNA-IQ™ (Promega) extracted on the Maxwell 16® minirobot (Promega). The PBSO Forensic Biology Unit conducted blood stain extraction protocols following manufacturer's protocols. All DNA samples were quantified using the real-time QuantiFiler kit from Applied BioSystems and DNA samples were concentrated using the Microcon-100 (Millipore, Billerica, MA) to ~15 ng/µL. Amplification was conducted at PBSO using the PowerPlex® 16 BIO kit (Promega) followed by separation and allele detection using the Hitachi FMBIO II flatbed scanner system.[142] Extracted DNA samples were also shipped to Berkeley for analysis on the portable microsystem. All the extraction methods yield DNA samples with similar quality and quantity for full STR profiles on our PCR-CE microdevice. Ultimately, DNA-IQ™ using the Maxwell 16® minirobot was chosen as the DNA extraction method for the mock crime scene study due to its consistent performance and automated process. The average DNA concentration obtained from blood stains on paper towels and cloth using the Maxwell followed by Microcon concentration is 16.8 ±1.4 ng/µL (n=6). In the mock crime scene, the DNA concentration added to the PCR reactor was estimated by this average concentration.

4.3.5 Mock crime scene setup and evidence collection

The PBSO Forensic Biology Unit prepared blood stain samples, representing a male and a female victim, and a male suspect, from known individuals with documented DNA profiles.[142] The suspect's DNA profile was entered into a mock CODIS "convicted offender" database at PBSO. Mock crime scene evidence was prepared in advance in the PBSO Forensic Biology Unit by placing liquid blood onto paper towels and a blue cloth shirt (50% polyester/50% cotton). Each stain was made by spotting one drop (3 µL) of each blood sample in several designated areas on the substrates using plastic disposable pipettes and then allowing the stains to dry overnight. The evidence was placed into separate paper bags and sealed for transporting to the mock crime scene at Lake Lytal Park in West Palm Beach, FL.

A PBSO Mobile Command Unit (MCU) was deployed to the mock crime scene, as shown in Figure 4.2. This MCU is equipped with a power generator, an air conditioning system, and a satellite internet connection, which are necessary for the on-site STR analysis. The DNA extraction equipment and materials were located in a separate room from the portable PCR-CE instrument to prevent interference between these two steps.

The crime scene was set up in a pavilion at Lake Lytal Park by PBSO crime scene investigators. Two body-shape cardboard dummy victims were laid on the ground. The blue cloth shirt (male victim blood stain) was placed on top of a dummy, one of the paper towels (female victim blood stain) was placed next to the dummies, and the other paper towel (male suspect blood stain) was placed on top of a park bench nearby the scene (Figure 4.2A and B).

Figure 4.2. Photographs of the mock crime scene.

The crime scene was investigated by PBSO in West Palm Beach, FL (A and B). Three blood stains on cloth or paper towels were laid out in the mock crime scene. (C) The entire DNA analysis was conducted in a mobile command unit provided by PBSO. (D) The portable forensic analysis instrument set up in the mobile command unit.

The crime scene was investigated as per Palm Beach County Sheriff's Office standard operating procedures. Crime Scene personnel recorded the scene using digital photography. Biological stains were collected by cutting them from the cloth and paper towels using scissors. All samples were packaged separately in labeled envelopes, documented on a PBSO property receipt and signed over to the on-scene DNA analyst in order to simulate the chain-of-custody procedures.

4.3.6 DNA analysis at mock crime scene

All DNA samples were extracted using the Maxwell 16® instrument with DNA IQ™ Casework Sample Kit (Promega) following the manufacturer's protocol. Briefly, an entire blood stain was removed from the envelope and placed in a labeled 1.5 mL microcentrifuge tube. A total of 175 µL of Incubation Buffer with Proteinase K (1.8 mg/mL Proteinase K and 0.1 M DTT (Dithiothreitol)) was added to each sample tube followed by 30-min incubation at 56 °C on a heat block. Then, 350 µL of Lysis Buffer was added to each tube. After vortexing and centrifugation, the lysates were released and separated from the substrate by placing the substrate in a spin basket inserted into the tube. The lysates (500 µL), as well as plungers and final tubes with 40-µL Elution Buffer, were manually loaded into a cartridge (DNA IQ™) on a rack placed in the Maxwell instrument. After the Maxwell run was completed in 30 min, DNA extracts in the final tubes were concentrated using Microcon columns to a final volume of 7 µL. Four microliters were used for the on-site analysis; the remaining sample and unprocessed blood stains were stored for possible future analyses.

Concurrently with DNA extraction, a microdevice for PCR-CE analysis was prepared as described previously. The PCR cocktail (4 µL of DNA sample in 10 µL of PCR cocktail) was loaded into the PCR reactor with a target concentration of 300 template copies in the 160-nL reactor. The PCR step was performed following the above described protocol (2 hr) and then the CE separations were performed 3 times (30 min). During thermal cycling, another microdevice for the next sample was prepared for streamlined operation.

4.4 Results and discussion

Crime scene genetic profiling must include successful DNA extraction, amplification, electrophoresis, allele detection and data interpretation. The portable PCR-CE microsystem must reproducibly amplify a multiplex STR system with a low limit of detection and accurately separate alleles with single base resolution. Since the objective is to generate a probative DNA profile which can be immediately searched in the convicted offender database, profiles obtained from real-time DNA typing should be compatible with FBI CODIS convicted offender database protocols. Additionally, the instrument must have simple operational protocols that allow the performance of on-site STR analyses that are consistent with current crime scene investigation procedures. The ability of the portable PCR-CE microsystem to meet these criteria has been assessed using a 9-plex autosomal STR system. The 9-plex system, consisting of amelogenin and 8 CODIS core STR, has a discrimination power (1.2×10^9 in U.S. populations) that is comparable with commercial typing kits,[10, 14] together with a shorter amplicon size range (106-259 bp) that provides a higher amplification efficiency and a shorter separation time.

4.4.1 Allelic ladder separation

The separation capability of the portable PCR-CE system for the chosen multiplex was first explored. Since the 9-plex STR system comprises the short amplicons (106-259 bp) of the PowerPlex® 16 system, we separated and analyzed the PowerPlex® 16 allelic ladder fragments in the 106-259 bp size range. To improve the fluorescent signal intensity, a preconcentrated PowerPlex® 16 allelic ladder (4×) was loaded into the PCR reactor, and co-injected with the ET550-R sizing standard into the 7-cm long CE channel for allele separation. The CE separation using this microdevice was completed in 8 min, which is approximately 20 min faster than the single-capillary ABI 310 instrument.[40] As shown in Figure 4.3 Panel A, the TH01 9.3 and 10 alleles, which differ by only one base pair, were distinguished with a resolution of 0.45. Alleles which differ by 2 bp in D21S11 were also successfully separated. Although the D13S317 and D8S1179 loci show weaker peak intensities resulting in imbalanced profiles, all the alleles in the 9-plex STR system were successfully resolved and sized, demonstrating that our microsystem can separate DNA fragments in the 106-259 bp range for forensic human identification. These results were used to generate the bin information in the MegaBACE™ program for designating the alleles in the subsequent STR analyses. Higher separation resolution can be readily achieved by simply employing a longer separation channel in the microchip design at the expense of slower separations or by using improved gel matrices.[86, 103] However, we elected to use the 7-cm channels in this study because of our focus on rapid point-of-analysis operation.

4.4.2 Standard DNA test

The 9-plex autosomal STR amplification and separation on the portable PCR-CE microsystem was demonstrated using standard female 9947A and male 9948 genomic DNA. Figure 4.4 shows representative STR profiles of each sample amplified from 100 template copies in the PCR reactor. Each standard DNA template was analyzed in triplicate in independent amplifications and separations using the same conditions. All the alleles in each analysis were resolved, balanced, correctly sized and designated using the MegaBACE™ program.

In the co-injection structure used here, PCR amplicons and sizing standards are injected into the injection channel simultaneously. To evaluate the sizing calibration accuracy, the run-to-run standard deviations (n=3) of the allele sizes were calculated from the traces of 9947A and 9948 DNA obtained in the standard DNA test. As shown in Figure 4.4 C and D, all the alleles can be sized correctly with a standard deviation $\leq$ 0.8 bp. Although the D13S317 alleles in the 9947A DNA analyses show a higher deviation (0.8 bp) due to their breadths, there is little injection mobility bias in the new injection structure demonstrating that our microsystem provides the necessary allele calling accuracy for human identification. Some loci, such as TH01, D7S820, and D8S1179, tend to show n-1 or n+1 stutter peaks. This is due to the high concentration of DNA templates (~1.88 ng/μL) in the 160-nL PCR reactor, compared to that recommended for commercial STR typing kits (0.02-0.04 ng/μL).[38] Off-chip amplifications confirmed that this is the cause of the observed stutter peaks (data not shown). This minor problem is being addressed through improved injection methods.

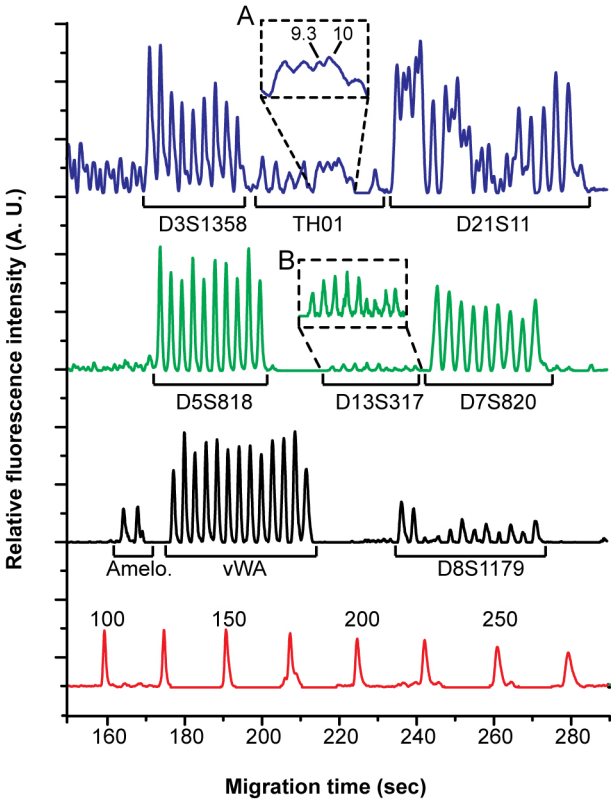

Figure 4.3. The 9-plex STR allelic ladder separation obtained on the portable PCR-CE microsystem.

(A) The TH01 locus trace has been expanded to show the 9.3 and 10 alleles which exhibit a resolution of 0.45. (B) The D13S317 locus is similarly expanded for display. D3S1358, TH01, and D21S11 loci are labeled with FAM (first trace). D5S818, D13S317, and D7S820 are JOE (second trace). Amelogenin, vWA, and D8S1179 are TMR (third trace). The sizing standard (fourth trace) is MegaBACE^{TM} ET550-R, labeled with the FAM-ROX energy-transfer dye.

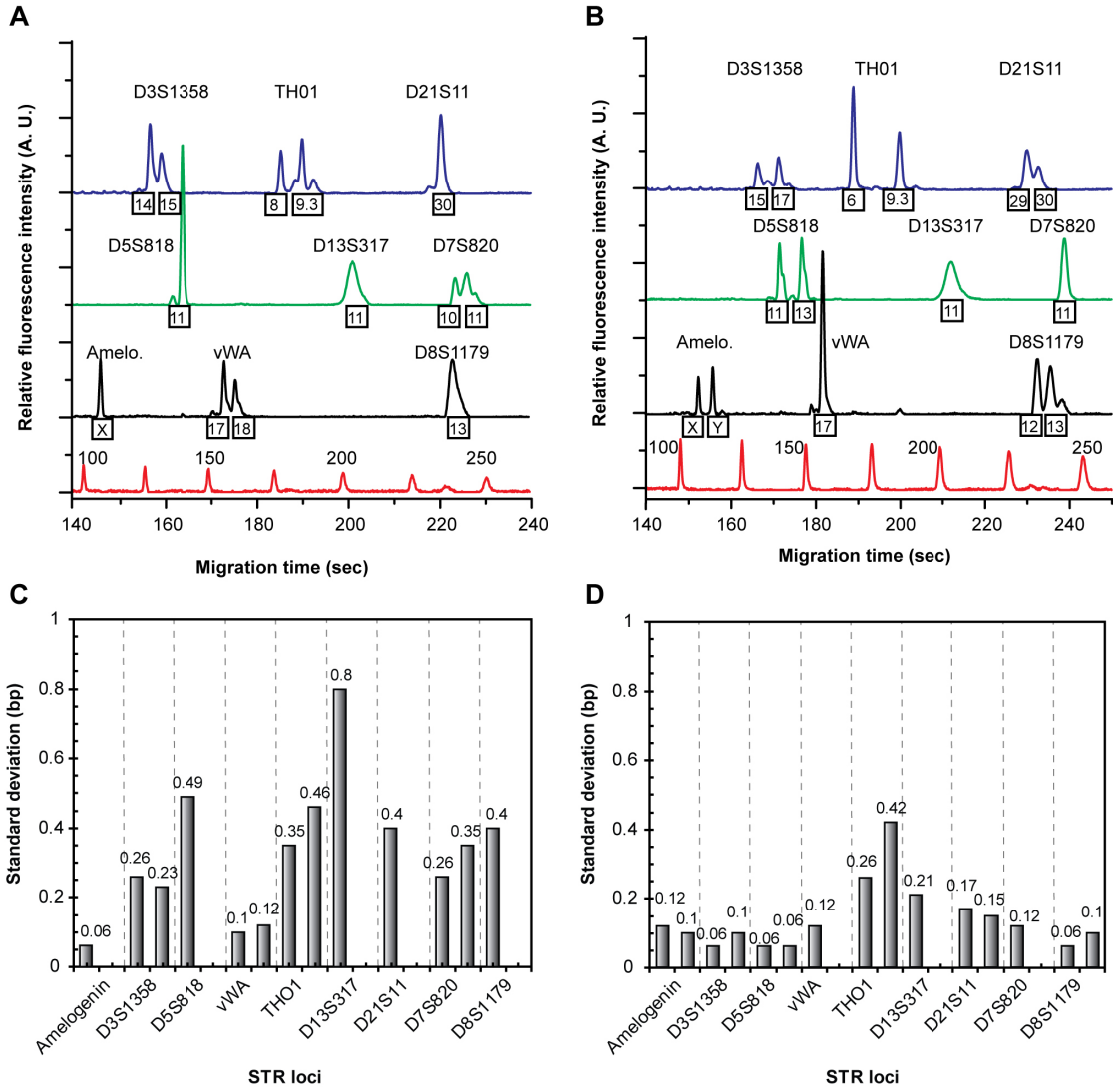

Figure 4.4. Standard genomic DNA analysis on the microdevice.

Representative 9-plex STR profiles of (A) 9947A female and (B) 9948 male standard DNA obtained with 100 copies of DNA template in the PCR chamber. The run-to-run standard deviations of the allele sizes for (C) 9947A and (D) 9948 DNA amplified and co-injected with sizing standards (ET550-R) using the PCR-CE microdevice on the portable instrument. Each sample was tested 3 times in independent amplifications and separations.

4.4.3 Limit of detection study

The sensitivity of the portable PCR-CE microsystem was evaluated using the 9-plex STR samples amplified from serially diluted 9947A standard DNA (200, 100, 50, 20, 10 copies of templates in the PCR chamber). Figure 4.5 shows the average percentages of full 9-plex STR profiles of 9947A DNA obtained from 3 runs at each DNA concentration as a function of input DNA template in the PCR reactor. Complete profiles (100% profile percentage) can be reproducibly obtained from 100 template copies. As the template concentration decreases (<100 pg or <33 copies),[27] imbalance within two heterozygous alleles occurred, causing allele dropout. As shown in Figure 4.5, when the DNA concentration was lowered to 50 copies, two full and one partial profile with one allele dropout were obtained, producing a 94.9% profile. With only 10 copies, 61.5% of the alleles are successfully amplified and detected. From the limit-of-detection study, the minimal template concentration needed to reliably produce complete DNA profiles was 100 template copies in the PCR reactor. We also found that excessive DNA concentration (>500 copies) results in split peaks or massive stutter peaks (data not shown).

4.4.4 DNA analysis at a mock crime scene

DNA analysis was performed at the mock scene in a Mobile Command Unit (MCU) provided by PBSO. The MCU was set up following the National Quality Assurance Standards.[143] The evidence handling and DNA extraction were conducted in a separated room from PCR-CE analysis. Although PCR preparation and post-PCR analysis were performed in the same room, neither sample-to-sample nor analyst-to-sample DNA contamination was detected. Finally, all allele calls were reviewed and verified by a qualified forensic DNA analyst to eliminate transcriptional errors.

In the field trial, we arrived in Lake Lytal Park at 7:00 am and the mock crime scene was set up in 10 min. At 7:10 am, the mock crime scene was investigated and the samples were collected in 20 min by a crime scene investigator from PBSO. At 7: 30 am, DNA extraction was initiated on the Maxwell system and completed in two hours. From 9:30 to 10:00 am the PCR cocktail containing the male suspect DNA sample (blood on a paper towel from the park bench) was prepared and loaded into the PCR-CE microdevice. The STR analysis on the portable instrument began at 10:00 am. The PCR reaction was complete in 2 hours, and the electrophoretic trace was obtained within 30 min. At 12:30 pm, the male suspect profile was reviewed and submitted by email to PBSO to be searched against the mock CODIS convicted offender database. The DNA profile from the crime scene was matched to an "offender profile" previously entered in the database. This information was relayed back to the crime scene within twenty minutes. *From sample collection to the generation of the CODIS hit, the entire process took only six hours.* Between 1:00 pm and 6:00 pm, the blood stains from the male (shirt) and the female victim (paper towel) samples were also typed on the portable instrument. These DNA profiles were successfully obtained and correctly typed at 3:30 pm and 6:00 pm, respectively. Figure 4.6 presents the electropherograms of the three samples analyzed with the 9-plex STR system. These results demonstrate that the portable PCR-CE system can be used at a crime scene to perform successful real-time STR typing without interfering with the crime scene investigation process.

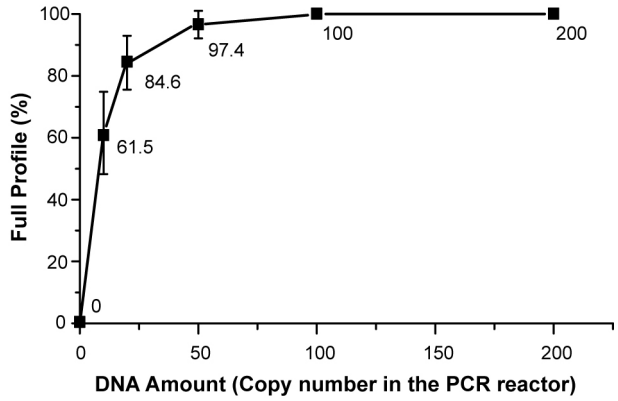

Figure 4.5. The characterization of the limit of detection.

Percentage of full 9-plex STR profiles obtained from 9947A standard DNA on the portable microsystem as a function input DNA. With 100 template copies in the PCR reactor, full profiles are reproducibly obtained.

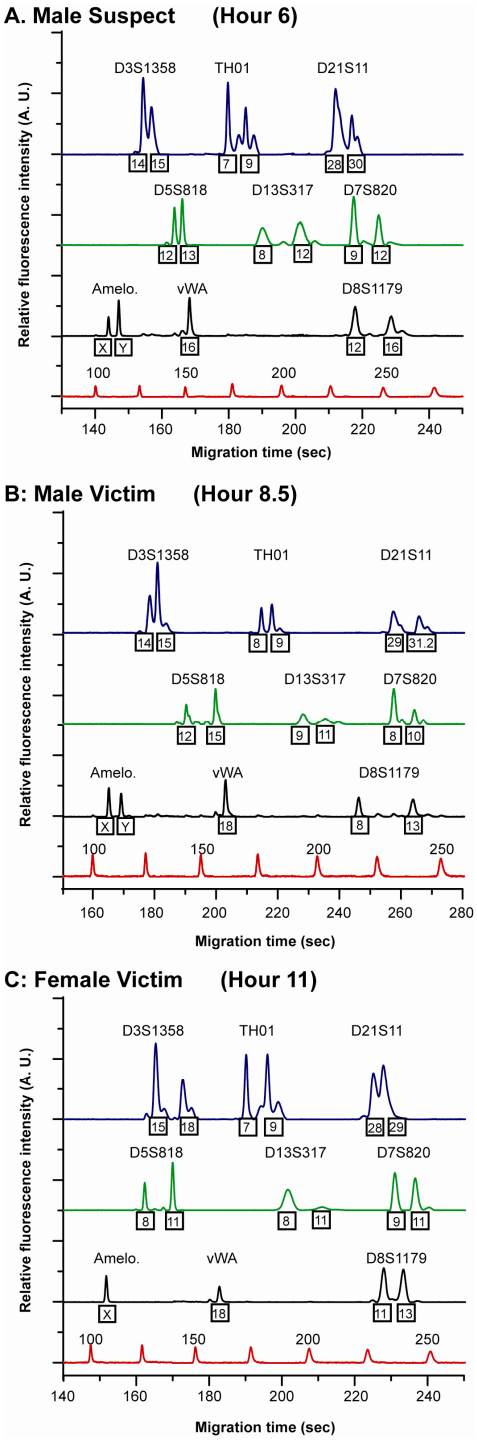

Figure 4.6. STR profiles from mock crime scene samples using the portable microsystem.

Within 11 hours, three blood stain samples, male suspect on a paper towel, male victim on cloth, and female victim on a paper towel, were successfully amplified and correctly typed. The suspect's profile was searched against the CODIS database and a hit was obtained at hour 6.

The utility of DNA analysis on probative biological material and a subsequent database search for a suspect depends on the timeliness of processing the material recovered from crime scenes.[144] Following current procedures, it frequently takes about 3 days from evidence collection to CODIS hit generation for a rush case. By conducting DNA analysis at a crime scene as demonstrated in this study, a CODIS hit can be obtained within six hours. This successful demonstration of on-site DNA typing validates the feasibility of real-time forensic human identification as well as the utility of identifying a suspect rapidly after a crime has been committed

Our portable PCR-CE microsystem is best utilized as a presumptive identification tool to provide probable cause in the apprehension of potential perpetrators whose profiles have previously been entered into a database, such as convicted felons. Because of the high frequency of recidivism (over 66%),[145] this is a very reasonable strategy. In this context, the use of 9-plex STR system is not limiting although with further device and process improvements, extension to 16-plex is possible. Due to the limited throughput of the current portable microsystem, only three blood stain samples were analyzed consecutively, and no positive and negative controls were included in the field trial. We recognize that a multi-lane microdevice that can process 4-6 samples in parallel is desirable. Laboratory versions of an integrated 4-channel PCR-CE microdevice have already been demonstrated.[73, 96] Furthermore, an affinity-capture-based in-line injector in place of the inefficient cross-injector in the current microdevice should achieve >10-fold detection sensitivity improvement.[77, 146]

The development and successful utilization of this portable forensic analysis system will initiate an important policy discussion about the possible uses of real-time forensic analysis. While defense and military applications of this technology are evident, the deployment of this portable forensic analysis capability in the domestic arena requires careful evaluation. We envision two possible applications for the purpose of discussion. First, our system could be used for real-time analysis at crime scenes where biological material could only have been left by the perpetrator, to advance case detectives' investigation before the suspect has fled the area or destroyed secondary evidence. Second, in states that require DNA samples at arrest, the ability to rapidly type an arrestee before release on bail might generate probable cause to hold the individual because of hits on other earlier criminal activities.

4.5 Conclusions

We have developed an integrated PCR-CE microdevice together with a compact portable instrument for real-time forensic human identification. A 9-plex autosomal STR multiplex is also demonstrated that provides 2.5-hour analysis time, 100-copy sensitivity, and ≤ 0.8 bp allele sizing accuracy. A mock crime scene was investigated following standard procedures, and three blood stain samples were successfully amplified and correctly typed at the scene. The suspect's profile was searched against a "mock" CODIS database and a hit was obtained in only six hours. This study is a significant step towards a fully integrated and portable forensic analysis system, enabling rapid real-time human identification.

Acknowledgements

We thank all the people involved in the mock crime scene project at the Palm Beach County Sheriff's Office, including Jeffrey A. Andrews, Kenneth Morrow, Benjamin A. Perillo, Sharon I. Rachlin, Ray Carlson and George H. Staley. We thank Susan Greenspoon at the Virginia Department of Forensic Science for valuable discussions. We also thank Promega Inc. for providing information about the PowerPlex® 16 STR system. The microdevice was microfabricated at the University of California, Berkeley Microfabrication Laboratory. This project was supported by Grant No. 2004-DN-BX-K216 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice. R.A.M. discloses a financial interest in Microchip Biotechnologies, Inc. (Dublin, CA) that is commercially developing aspects of the technologies presented here.

Chapter 5

Fully Integrated Genetic Analyzer for Forensic DNA Typing

5.1 Summary

A fully integrated micro total analysis system for forensic short tandem repeat (STR) analysis has been developed that includes sequence-specific DNA template purification, polymerase chain reaction (PCR), post-PCR cleanup and capture inline injection, and capillary electrophoresis (CE). Fragmented genomic DNA is hybridized with biotin-labeled capture oligos and pumped through a fluidized bed of magnetically immobilized streptavidin-coated beads in microchannels where the target DNA is captured. The bead-DNA conjugates are then transferred using an on-chip micropump to a 250-nL PCR reactor comprised of a microfabricated PCR heater and a temperature sensor for autosomal STR amplification. The resulting PCR products are electrophoretically injected through a streptavidin-modified capture gel where they are bound to form a concentrated and purified injection plug. The thermally released sample plug is injected into a 14-cm-long CE column for electrophoretic separation and detection. The DNA capture efficiency provided by the on-chip sequence-specific DNA template purification is determined to be 5.4% using K562 standard DNA. In initial experiments standard DNA (80 ng) was successfully analyzed on this microsystem and a full STR profile obtained in about 2.5 hr. This fully integrated microsystem with sample-in-answer-out capability is a significant advance in the development of rapid, sensitive, and reliable micro total analysis systems for rapid human identification.

5.2 Introduction

Forensic scientists always strive to find techniques that can increase the throughput, lower the cost, and improve the reliability of forensic short tandem repeat (STR) analysis.[9, 147] Microfabricated bioanalysis devices have the potential to address this challenge by providing facile scaling capability, lower reagent consumption, and the ability to integrate multiple analytical steps on a single device.[41, 42, 148] Significant advances have been achieved toward developing microdevices on which individual STR typing steps, i.e. DNA extraction,[112, 113, 149] polymerase chain reaction (PCR),[58, 136, 150] or capillary electrophoresis (CE),[103, 104, 137] can be carried out. Although these chip-based analyses provide better performance over conventional methods, they are still primarily utilized by the academic research community, because their high dependence on other off-chip processes makes the adoption of these new technologies less attractive to forensic laboratories.

The most valuable advantages provided by micro total analysis systems (μTAS) stem from their integration capability. Performing the entire DNA typing process in a single microsystem decreases the reagent and time consumption, makes the process more automated and robust, and reduces sample handling by users, which can eliminate the risk of sample mix-up and contamination. The Mathies group has reported several pioneering studies towards the integration of sample processing steps, such as PCR with microfabricated electrophoresis systems for forensic STR typing.[58, 105, 106] They have developed an integrated PCR-CE microdevice as well as a portable detection instrument for on-site rapid human identification.[105] Real-time DNA analyses at a mock crime scene, including sample collection, DNA extraction, STR analysis on the PCR-CE microsystem, and CODIS database search, have been successfully conducted in collaboration with law enforcement agencies.[106] While this demonstration validates the feasibility of STR typing using integrated devices, this microsystem falls short in its ability to analyze real-world samples containing PCR inhibitors; thus, integrating a DNA purification technique prior to the analysis is necessary.

Several fully integrated microsystems with sample-in-answer-out capability have been developed for genetic analysis.[54, 68, 72, 100] One of the most promising systems which could be used for forensic STR typing was presented by the Landers group.[72] They successfully integrated a solid-phase extraction (SPE) column for DNA extraction with PCR and CE on a single device for pathogen detection. However, the delicate operation and low efficiency of the pressure-driven cross injection for CE separation and the transfer of purified DNA from the SPE column to the PCR reactor might be problematic for analyzing forensic samples, which have a great disparity in sample quality and quantity.[92]

To address the low efficiency associated with the conventional cross injector for CE separation, researchers have invented various preconcentration and inline injection methods.[74, 78, 97] Yeung et al. successfully applied this concept into forensic STR analysis by developing an integrated STR sample cleanup, capture inline injection, and CE separation microdevice for high-sensitivity DNA typing.[81] The integration of this capture inline injector into a fully integrated microsystem should significantly advance the system sensitivity and reliability. Furthermore, the near 100% injection of all of the thermally cycled products will give the microsystem quantitative analysis capability.

Sample transfer from the DNA extraction process to PCR is more troublesome, because the process involves both sample transportation and buffer exchange from extraction buffers to

PCR solutions. Magnetic beads are an excellent sample transfer medium in an integrated microsystem because they can be precisely manipulated using an external magnet facilitating washing and buffer exchange. Several commercial DNA extraction systems that make use of magnetic particles, such as ChargeSwitch® and DNA IQ™, have been used for forensic STR analysis, and have the potential to be translated into chip formats.[35] However, the DNA binding in these systems require PCR-incompatible solutions, such as chaotropic salts or acidic solutions. Moreover, the buffer exchange from DNA binding buffers to PCR solutions could cause severe sample loss as PCR solutions readily release DNA from these particles.

Magnetic beads modified by oligonucleotide probes are capable of capturing specific DNA templates via DNA hybridization from a complex cellular background.[151-153] This method is attractive for DNA extraction in a fully integrated microsystem because no harsh chemicals are employed during DNA binding and the release of the captured DNA from magnetic beads can be effectively controlled by on-chip heating instead of buffer exchange. In addition, since only DNA of interest is captured for the subsequent PCR and background DNA sequences are effectively eliminated, the PCR efficiency could be significantly improved. This method has been successfully utilized for the extraction of plasmid DNA and genomic DNA fragments from clinical samples, but no work related to forensic STR typing has been reported yet.[153-155]

The work presented here demonstrates the successful integration of the novel sequence-specific DNA extraction and the improved post-PCR capture inline injection into the well-characterized PCR-CE system to form a fully integrated microdevice for forensic STR analysis. Using the bead capture structure developed previously,[75, 101] a fluidized bed of streptavidin-coated magnetic beads captures the conjugates of biotin-labeled oligonucleotide probes and genomic DNA fragments containing the STR locus sequences. After DNA capture, the bead-DNA conjugates are pumped to a PCR reactor for 9-plex STR amplification. The resulting biotin-labeled PCR products are electrophoretically driven through a streptavidin-modified capture gel where they are bound and concentrated into a narrow injection plug, followed by thermal release for CE separation. By integrating these components on a single chip, we automate the process, improve the reliability, and minimize the risk of contamination during the sample analysis. This structure has enabled the successful STR typing from forensic samples in a fully integrated microdevice.

5.3 Materials and Methods

5.3.1 Microdevice design

The microdevice shown in Figure 5.1 contains two identical genetic analysis systems forming a symmetrical doublet on a 4-inch glass wafer. The structure is similar to the device developed in our group previously,[101] but the design is modified to integrate the post-PCR cleanup and inline injection functions and to adapt to the newly developed scanner instrument. Each analytical system includes a poly(dimethysiloxane) (PDMS) micropump and two PDMS microvalves[122] for fluidic control, a 4-cm-long bead capture structure with a system of bifurcating channels for DNA template capture,[75] a 250-nL PCR chamber with a microfabricated heater and a resistance temperature detector (RTD) for PCR thermal cycling, a 500-µm-long double-T channel junction with a tapered structure for post-PCR cleanup and inline injection,[81]

and a 14-cm-long channel for CE separation. These two systems share an anode, a cathode, and a waste well to reduce the number of reservoirs on the chip.

The microdevice is constructed using a four-layer wafer stack consisting of (from top to bottom) glass manifolds, PDMS membranes, a glass fluidic wafer, and a glass RTD wafer. The microfabrication process has previously been described in detail.[101, 105] Briefly, to form the fluidic layer, a 500-µm thick Borofloat glass wafer is coated with 2000-Å amorphous silicon on one side and 200-Å Ti and 2000-Å Pt on the other side. The PCR and CE pattern is etched to a depth of 40 µm while the bead capture structure is etched 30 µm deep on the amorphous silicon side using a 49% hydrofluoric acid (HF) bath. Next, the channels for the on-chip micropump are patterned on the Ti-Pt side of the same wafer. After etching the exposed Ti-Pt layer using hot aqua regia, the channels are etched to a depth of 30 µm using HF. The PCR heaters are also microfabricated on the Ti-Pt side of the fluidic wafer by electroplating gold leads followed by ion beam etching of Ti-Pt for heating elements. Finally, holes are drilled using a CNC mill for via holes, fluidic reservoirs, as well as electrical access holes.

To form the RTD wafer, a 762-µm Borofloat glass wafer coated with 200-Å Ti and 2000-Å Pt is patterned and etched using a hot aqua regia bath. The RTD wafer and the fluidic wafer are thermally bonded in a vacuum furnace at 650 ^{o}C for 6 hr. The glass manifold is fabricated from a 700-µm Borofloat glass wafer using the same method as above. The on-chip pumps and valves are assembled by sandwiching a PDMS membrane between the device and the manifold. A glass microchip which only contains a PDMS micropump and bead capture microchannels is also fabricated using the same method as described above to study the DNA capture efficiency.

Prior to use, the microchannels are coated with 0.25% polyDuramide (pDuramide) dynamic coating polymer to minimize DNA absorption to the channel walls and electroosmotic flow during electrophoresis. The coating procedure consists of 1 M HCl incubation for 15 min, DI water flush, and pDuramide incubation for one hour. After treatment, the chips are flushed with water again, and then dried with vacuum.

5.3.2 Scanner detection instrument

The instrument used to perform analyses with the microdevice is shown in Figure 5.2. The instrument contains a 488-nm diode laser (75 mW, Sapphire 488-75, Coherent, Santa Clara, CA), an optical system with a rotary objective for detecting four different fluorescence signals, pneumatics for the on-chip PDMS microvalves, electronics for PCR temperature control, and four high voltage power supplies for CE. The analysis system has dimensions 12 × 12 × 8 in., which can be used as a either bench-top or portable instrument. A LabVIEW graphical interface (National Instruments, Austin, TX) developed in-house is used to control the system through a DAQ board (NI 6259, National Instruments).

The schematic of the four-color confocal detection system is similar to the scanners developed in our group previously,[103, 156] but the optical components have been rearranged in order to fit into a limited space in the instrument. Briefly, the laser beam is reflected by mirrors, passes though a dichroic beamsplitter (z488bpxr, Chroma, Brattleborro, VT) and a hollow shaft stepper motor (U17-7, Empire Magnetics, Rohnert Park, CA), and is focused into the channel in the microdevice with a custom-built objective (Coastal Optical systems, Jupiter, FL) mounted on a rhomb assembly. The objective scans the microchip at 5 Hz with a radius of 7 mm. The returning fluorescent signal is collected by the objective and reflected by the beamsplitter into a confocal assembly where the light is focused on 200 um pinhole, and collimated into a 1 mm

diameter beam that enters the 4-color PMT (Hamamatsu H9797, Bridgewater, NJ). The converted electrical signals are processed using a 5-Hz low-pass filter and collected at a rate of 5000 data points per revolution of the objective using the 16-bit DAQ board.

During operation, the microdevice is placed onto a 6" heating stage on the top of the instrument and held in place with a plexiglass manifold as well as vacuum supplied by the instrument. The manifold contains spring-loaded pins pressed against the electrical pads on the device, providing the connections for sensing the RTD and powering the PCR heater. The manifold also contains Pt electrodes that are positioned within the reservoirs on the microchip for the application of high voltages during electrophoresis.

The design of the electrical circuits for driving the RTD and heater is the same as presented eariler.[64] Temperature control is accomplished through a proportion/ integrator/ differentiator (PID) module in the LabVIEW program. The PDMS microvalves are controlled using vacuum or pressure supplied through pneumatic connections to the valve access holes on the glass manifold. Twenty-one pneumatic lines are available for fluidic control. Each line can be switched between vacuum and pressure using a solenoid valve (LHLX0500200BB, The Lee Company, Essex, CT) controlled through the DAQ board.

5.3.3 STR typing and DNA capture probes

A 9-plex autosomal STR typing system was developed previously based on the primer sequences and fluorescence dye labeling scheme used in PowerPlex® 16 System (Promega, Madison, WI).[81, 105] To enable the post-PCR cleanup and inline injection, the unlabeled primers were replaced with biotin-labeled primers (IDT, Coralville, IA). The STR loci included in the 9-plex system are amelogenin for sex typing and 8 CODIS core STR loci (D3S1358, TH01, D21S11, D5S818, D13S317, D7S820, vWA and D8S1179). Primer sets without any labeling were also synthesized by IDT for DNA quantitiation using real-time PCR.

The PCR mixture prepared for on-chip 9-plex STR typing is comprised of 1.5× Gold ST*R buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 µg/mL BSA, 200 µM each dNTP) (Promega, Madison, WI), the primer mixture, 0.12 U/µL FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN), and deionized water.

The biotin-labeled primers in the 9-plex STR typing systems were also employed as capture probes for DNA template capture prior to PCR. The concentrations of the capture probes in a 10× capture probe mixture are listed as follows: Amelogenin: 27 nM, D3S1358: 17 nM, TH01: 22 nM, D21S11: 43 nM, D5S818: 11 nM, D13S317: 13 nM, D7S820: 47 nM, vWA : 13 nM and D8S1179: 11 nM. The capture probe mixture was incubated with genomic DNA for liquid-phase DNA hybridization, and then captured by streptavidin-coated magnetic beads via streptavidin-biotin binding on the chip.

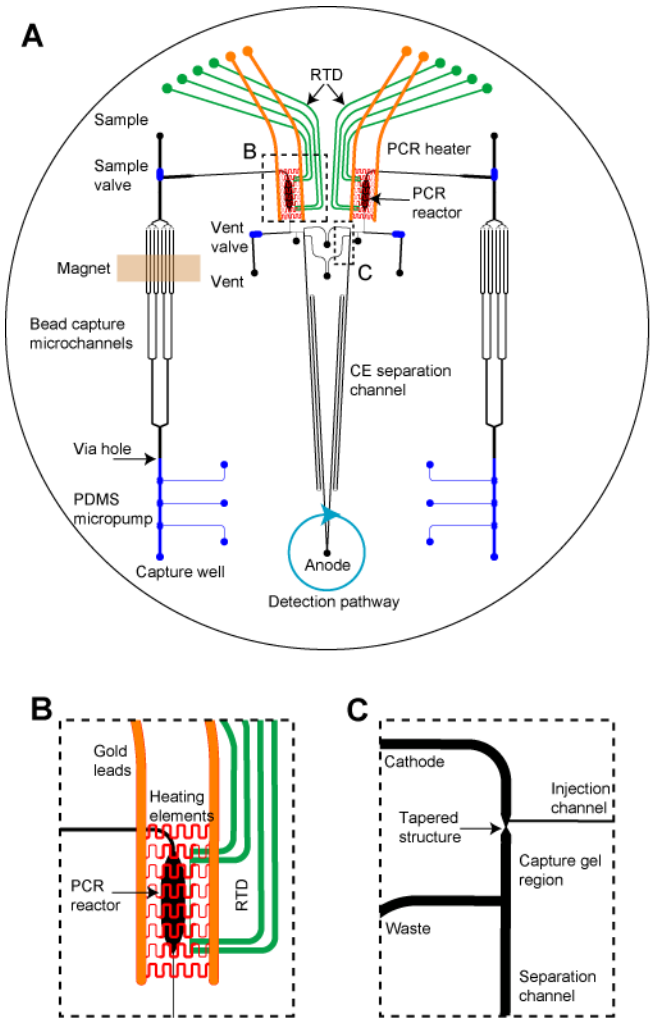

Figure 5.1. The design of the fully integrated microdevice for forensic STR analysis.

(A) Mask design for the microchip capable of performing DNA template capture, PCR, capture inline injection and CE separation. The microchannels are indicated in black, the microfabricated RTD and electrodes are in green, the heater is shown in red, the gold leads of the heater are drawn in gold, and the PDMS micropumps and microvalves are in blue. (B) Expanded view of the PCR chamber and the microfabricated heater and RTD. (C) Expanded view of the capture inline injector with a tapered structure.

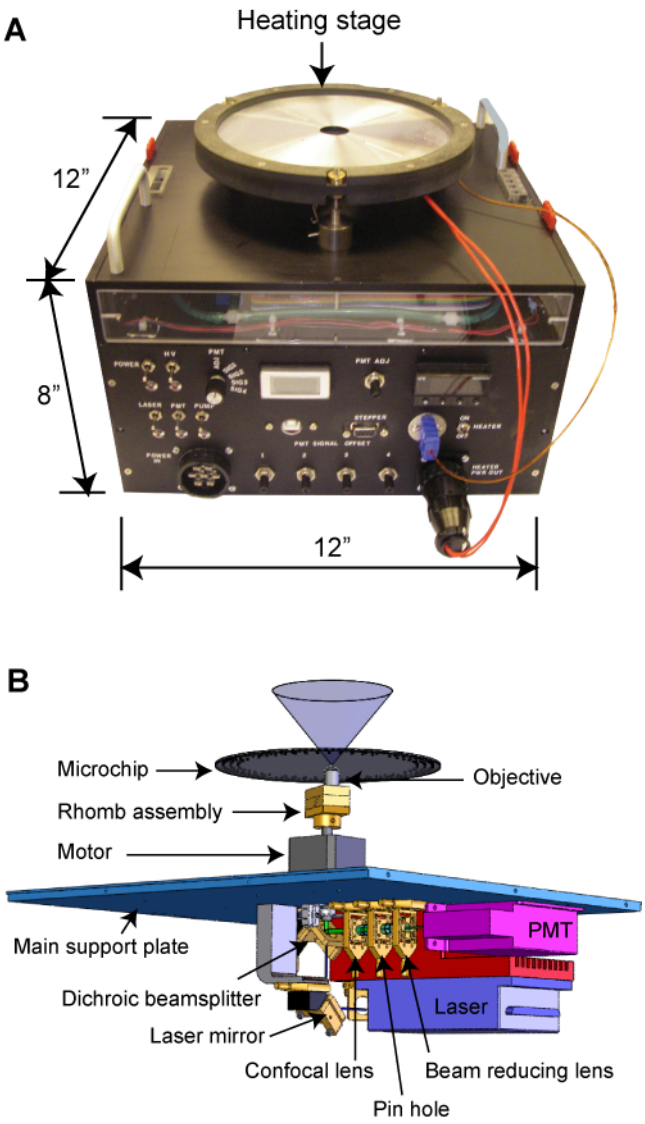

Figure 5.2. The structure of the scanner system for chip operation and detection.

(A) Photograph of the scanner. The analysis system has dimensions 12 × 12 × 8 in. (B) Solid-work view of the four-color confocal fluorescence detection system. (This instrument is designed and fabricated by Dr. James R. Scherer. The figures are also provided by Dr. Scherer.)

5.3.4 DNA sample preparation

Standard genomic DNA 9947A, 9948 and K562 were purchased from Promega and diluted in deionized water (DI water). To determine the heating time for fragmenting genomic DNA, K562 DNA (0.4 µg/µL) with high molecular weight was subjected to 95°C heat incubation for different times (from 10 to 40 min in 5-min intervals) in a PTC-200 thermocycler (MJ Research, Waltham, MA). DNA samples are then run on a 1.2 % agarose gel with 100-4 kb and 1-4 kb sizing ladders (Lonza, Allendale, NJ). These results demonstrate that 20-min heating is sufficient to fragment the genomic DNA into a size range of 1-4 kb. To prepare DNA samples for on-chip template capture, 20 µL DNA samples were first heated at 95 ^{o}C for 15 min in a PTC-200 thermocycler. After mixing the fragmented DNA with 25 uL of 20× sodium saline citrate (SSC) buffer and 5 uL of 10× capture probe mixture, the solution was further heated to 95 ^{o}C for 5 min, followed by incubation at 50 ^{o}C for 20 min to allow DNA hybridization between the biotin-labeled capture probes and the target DNA fragments.

5.3.5 Sequence-specific DNA template capture

A commercial streptavidin-coated magnetic bead system, Dynabeads M-280 (2.8 µm diameter, 6×10^5 beads/µL, Invitrogen, Carlsbad, CA) was used for on-chip sequence-specific DNA template capture following a modified protocol from Invitrogen. 10 µL beads were washed two times and resuspended in 25 µL 10× SSC solution for the on-chip DNA template capture experiment.

The evaluation of the efficiency of the DNA template capture was performed on the glass microchip which only contains bead capture microchannels coupled with a PDMS micropump. The operation procedure is as follows: the microchip is first filled with a 1% w/v solution of bovine serum albumin (BSA) and incubated for 15 min to block non-specific bead adhesion to the channels. Next, acetonitrile (ACN) is drawn by vacuum into the channel to eliminate any bubbles in the system followed by rinse with DI water. 10× SSC is finally loaded into the bead capture channels to serve as a running buffer for the subsequent DNA capture.

The DNA template capture process begins by introducing 5 µL of prepared Dynabeads solution (~1.3×10^6 beads) into the capture well. The magnetic beads are driven into the capture structure using the on-chip micropump and immobilized in the microchannels using a nickelplated neodymium magnet (All Electronics, Van Nuys, CA, MAG-74). A multi-step loading procedure is employed to establish equal bead distributions across all parallel capture channels.[75] DNA solution (20 µL) containing fragmented genomic DNA and capture probes is then pumped through the capture structure using a 9-step pumping protocol with three "flutter" steps (200 ms/step for pumping and 100 ms/step for fluttering).[75, 101] Following sample loading, 20 µL 1× Gold ST*R PCR buffer (Promega) is rinsed through the capture channels and beads to eliminate unbound material from the system. Finally, the entire bead bed is pumped out of the channels to a reservoir and collected for subsequent DNA quantitation.

To quantify DNA capture by the Dynabeads, real-time PCR is performed on ABI 7300 instrument (Applied Biosystems, Foster City, CA) using a SYBR® Green PCR Master Mix (Applied Biosystems). Each locus in the 9-plex STR typing system is quantitated separately. 2 µL non-labeled primer sets and 2 µL DNA template captured on the beads are mixed with the PCR Master Mix and DI water to a final volume of 25 µL according to the manufacturer's

protocol. DNA quantity is determined by a calibration curve generated using serially diluted standard DNA.

5.3.6 Streptavidin capture gel preparation

A 500 µL streptavidin gel solution, containing 5 % (v/v) bis-acrylamide (19:1, Bio-Rad, Hercules, CA), 8 M Urea, 1x TTE (500 mM Tris, 500 mM TAPS acid and 100 mM EDTA), 2 µg/µL streptavidin-acrylamide (Invitrogen), 0.0006 % riboflavin (w/v) and 0.125 % TEMED (v/v), is prepared in an opaque 2-mL scintillation vial with Teflon closure (National Scientific, Rockwood, TN) following the method developed previously in our group.[81] To form a capture gel, a 5% linear polyacrylamide (LPA) gel is first pushed into the channel from the co-injection well to just reach the injection channel using a syringe. The function of the gel is to protect the PCR reactors from being contaminated by the streptavidin gel solution which contains PCR inhibitors, such as acrylamide monomer and EDTA. Using a UV exposure setup installed on a Nikon inverted microscope,[80, 81] a 500-µm capture gel plug is formed in the double-T channel junctions.

5.3.7 Chip operation

Following the photopolymerization of the capture gel plugs in the chip, a separation matrix (5% LPA with 8 M Urea in 1× TTE) is loaded from the anode to the waste and from the cathode to the coinjection reservoir to form a matrix-capture-matrix gel sandwich structure in the capture inline injection regions. The tapered structure in the capture region ensures that the capture gel plug will be retained. After gel loading, a 15 min incubation with 1% w/v BSA solution followed by acetonitrile rinse is conducted. SSC (10×) is finally loaded into the bead capture channels while keeping the vent microvalve closed to prevent SSC buffer from getting into the PCR reactors.

The bead loading and DNA template capture are performed following the procedure described above. During this process, the vent microvalve is closed and all waste flows out to the sample well, preventing any potential contamination of the PCR chamber. After DNA sample loading, 10 µL PCR cocktail containing all the necessary components for PCR except DNA template is used to wash the bead bed. The bead bed is then pumped into the PCR chamber by closing the sample valve, opening the waste valve, and placing the magnet above the reactor.

Once the magnetic beads are loaded into the PCR reactor, ten more microliters PCR cocktail is loaded into the capture well and pumped through the beads. After that, the PCR thermal cycling begins with all valves held closed at 20 kPa. The modified thermal cycling protocol starts with an initial activation of the Taq polymerase at 95 ^{o}C for 4 min. For the next 32 PCR cycles, the temperature is held at 94 ^{o}C for 10 s denaturing, then ramped to 58 ^{o}C for 20 s annealing, and then to 70 ^{o}C for 30 s extension. Finally, a post extension step is performed at 70 ^{o}C for 5 min. Total PCR time is 40 min.

To perform the purification, the sample valve is held open and the biotin-labeled PCR products are electrophoretically injected using an electrical field of 25 V/cm from the PCR chamber to the waste well through the capture gel plug. The products are bound via the biotin-streptavidin interaction to form a tightly concentrated plug in the capture gel. Unbound materials are washed away in the washing step. The fluorescently labeled DNA strands retained in the capture gel are then thermally released into the separation channel by heating the whole chip to 67 ^{o}C and applying an electrical field of 250 V/cm towards the anode. After each run, all the

gels and solutions in the chip are removed out with water and the channels and chambers are cleaned using piranha (7:3 H_2SO_4: H_2O_2) to prevent run-to-run carryover contamination.

5.4 Results and discussion

5.4.1 Microsystem integration

The integration of the entire STR analysis on a single microdevice is more than the simple combination of several microfabricated units.[134, 148] Not only must all units have high performance, the sample/product transport between each analytical step also needs to be convenient and efficient. These considerations are even more critical for forensic STR analysis, where high sensitivity and reliability are desired due to the great disparity in sample quality and quantity, as well as the probative value of these samples.

In a fully integrated microdevice designed for forensic STR typing, three sample transfer steps should be considered: macro-to-micro interface, DNA extraction to PCR, and PCR to electrophoresis. The fully integrated microsystem presented here has successfully addressed each of these issues. First, the capture of DNA-probe conjugates using a magnetic bead bed immobilized in the microchannels can concentrate DNA samples with various volumes and concentrations into the microdevice, serving as an efficient macro-to-micro interface for the microsystem. Second, to transfer sample from the DNA extraction step to PCR, magnetic beads were employed as a medium to carry DNA into the PCR reactor. During the introduction of the PCR solution into the microsystem, since no DNA release occurs, precise timing control is not required and the magnetic beads can be thoroughly washed with PCR solution to remove inhibitors. Third, a capture inline injection structure was integrated into the system to replace the inefficient cross injector for CE separation. These steps not only provide near 100% sample transfer efficiency so that high-sensitivity analysis can be achieved, they also eliminate the need for delicate timing control and voltage balance, making the overall operation error-proof and reliable.

5.4.2 Genomic DNA digestion

To enable the sequence-specific DNA capture, genomic DNA with high molecular weight must be fragmented into an appropriate size range which can be captured by magnetic beads while still providing intact templates for subsequent PCR amplification. Physical methods, including hydrodynamic shearing,[157, 158] nebulization,[159] and ultrasonication,[160] are the most extensively used methods for breaking chromosomes in DNA sequencing library construction. However, to generate DNA fragments with controllable and reproducible sizes, expensive, bulky instruments are often required. Restriction enzymatic digestion is another well-established method to fragment genomic DNA.[161, 162] But the enzyme reaction usually takes more than one hour to complete and requires specific buffer systems and can be expensive. DNA digestion by heating was chosen in this study because it can be rapidly performed in a conventional thermal cycler. Moreover, since the DNA extraction process in forensic sample analysis already includes a heating step for cell lysis, the heat digestion can be easily incorporated into the extraction procedure without any additional operation. As shown in Figure 5.3, heating DNA samples at 95 ^{o}C for 20 min is sufficient to fragment genomic DNA into a size range of 1-4 kb. Breaking DNA into small fragments will result in poor PCR efficiency, because it is more likely that DNA is

broken inside the target sequences. On the other hand, fragments longer than 4 kb will decrease DNA capture efficiency. Because the size range of STR alleles in the 9-plex STR typing system is 106-259 bp, 1-4 kb is a reasonable size range for the subsequent DNA capture and amplification steps.

5.4.3 Sequence-specific DNA capture

The DNA capture efficiency was characterized on a microchip that only contains bead capture channels and a PDMS micropump. Total 0.8 µg K562 standard DNA is first digested by heating at 95 ^{o}C for 20 min, followed by incubation with 1× capture probe mixtures at 50 ^{o}C for another 20 min. The resulting 20 µL DNA solution is then pumped through the magnetically immobilized beads (~1.3×10^{6} beads) in the channels at a flow rate of 1 µL/min using a 9-step pumping protocol.[75, 101] Following a bead washing step with 20 µL PCR buffer, the bead-DNA conjugates are pumped out and the captured DNA is quantitated by real-time PCR. The total on-chip operation, including sample loading and washing, takes about 45 min to complete. As demonstrated previously,[75, 101] factors which affect the on-chip DNA capture efficiency include capture channel design, pumping scheme, and flow rate. The structure of the capture channels, including channel number, channel depth and width, followed the design developed previously in our group to ensure optimal operation.[75, 101] The pumping scheme including pumping and flutter steps as well as the flow rate are considered together to maximize the capture efficiency while still keeping the operation as fast as possible. Previous studies demonstrated that a pumping protocol consisting of at least three flutter steps is critical for optimized capture efficiency because the pulsatile motion of the beads results in better contact with target DNA.[75] However, these extra flutter steps slow down the sample loading and can make the operation impractically long. Considering that a sample volume of 20 µL is common in forensic STR typing, the pumping steps were shortened to 200 ms to achieve a flow rate of 1 µL/min, resulting in a reasonable sample loading time of 20 min.

Since the capture probes may have different capture efficiency for different STR loci, the concentrations of these probes were iteratively adjusted to ensure balanced template capture for all 9 STR loci. As shown in Figure 5.4, the capture efficiencies for these 9 STR loci are in a range of 3.7 - 7.0 % with an average of 5.4 ± 1.3 %. Although this efficiency is lower than those of many stand-alone DNA extraction microdevices developed previously,[112, 113, 149] the 100% transfer of the captured DNA to the PCR reactor compensates for this drawback and makes the sequence-specific DNA capture suitable for high-sensitivity STR typing.

Another advantage provided by sequence-specific DNA extraction is the improved PCR efficiency due to the complete removal of background DNA sequences. Figure 5.5 presents 9-plex STR profiles amplified from 1 ng of whole genomic DNA (9948) and from ~ 1 ng DNA fragments purified from genomic DNA by sequence-specific DNA capture. The PCR amplifications were performed in a conventional thermal cycler with a PCR protocol which includes denaturation at 94 ^{o}C for 10 s, annealing at 58 ^{o}C for 20 s, and extension at 70 ^{o}C for 30 s. Compared to the PCR protocol recommended by the manufacturer, over 1.5 hr is saved.[38] Using this shortened cycling, full STR profiles still can be obtained from the purified DNA. In contrast, the profiles from the whole genomic DNA experienced dropout of the TH01 9.3 allele. This comparison demonstrates the effectiveness of sequence-specific DNA purification for improving STR amplification.

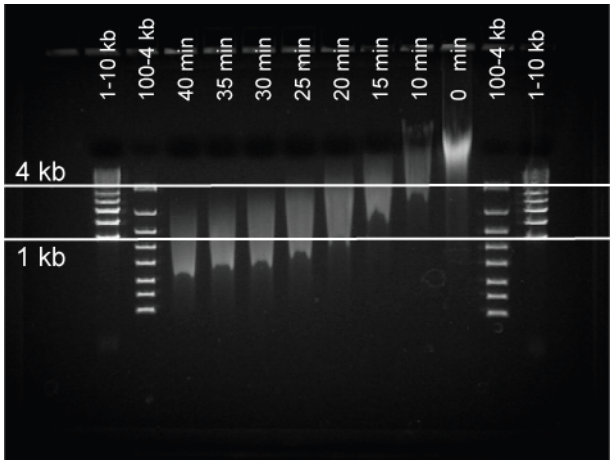

Figure 5.3. Photograph of gel electrophoresis separation of genomic DNA thermally fragmented for different lengths of time.

K562 standard genomic DNA with high molecular weight was subjected to 95°C heat incubation for different times and run on a 1.2 % agarose gel. 20-min heating is sufficient to fragment the genomic DNA into a size range of 1-4 kb.

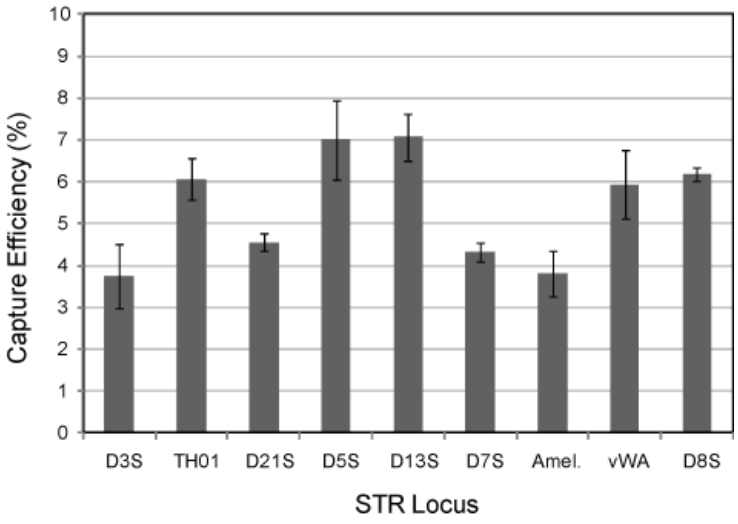

Figure 5.4. The capture efficiency of the sequence-specific DNA template purification process.

By optimizing the capture probe concentrations, similar capture efficiencies with an average of 5.4% were obtained for all 9 STR loci.

This improved PCR efficiency is extremely useful in the quest to expedite forensic STR typing. In current STR amplifications, high annealing temperatures, long holding time and slow temperature ramping rate are usually employed in order to ensure balanced and highly stringent amplifications of all STR loci and to avoid non-specific amplicons. For example, the Powerplex 16 STR kit recommends 60 ^{o}C for annealing and a temperature ramping rate of 0.5 ^{o}C/s from denaturation to annealing, resulting in a PCR protocol longer than 3 hours.[38] In our previous study of on-chip STR amplifications, we also found that the slow ramping rates are critical for generating reproducible and balanced STR profiles.[106] Faster temperature transitions resulted in allele dropout and imbalanced profiles. The on-chip PCR time was thus only shortened to 2 hours. Sequence-specific DNA template capture is an effective way to overcome this hurdle for rapid STR amplification. The removal of unnecessary DNA sequences eliminates the associated potential non-specific amplification, adding stringency prior to the PCR step. Under these conditions it is possible to use a lower annealing temperature allowing more efficient primer binding together with a faster ramp rate that accelerates the PCR process.

This improved PCR efficiency is extremely useful in the quest of expediting forensic STR typing. In current STR amplifications, high annealing temperatures, long holding time and slow temperature ramping rate are usually employed in order to ensure balanced and highly stringent amplifications of all STR loci without products of any non-specific amplicons. For example, Powerplex 16 STR kit requires 60 ^{o}C for annealing and a temperature ramping rate of 0.5 ^{o}C/s from denaturation to annealing, resulting in a PCR protocol longer than 3 hours.[38] In our previous study of on-chip STR amplifications, we also found the slow ramping rates are critical for generating reproducible and balanced STR profiles.[106] Faster temperature transitions resulted in stochastic allele dropout and imbalanced profiles. The on-chip PCR time was thus only shortened to 2 hours. Sequence-specific DNA template capture is an effective way to overcome this hurdle for rapid STR amplification. The removal of unnecessary DNA sequences eliminates the associated potential non-specific amplifications, adding stringency prior to the PCR step and allowing primers to efficiently bind to target sequences so that a lower PCR annealing temperature together with a fast ramping rate can be used to accelerate the PCR.

5.4.4 Standard DNA test

DNA template capture, 9-plex autosomal STR amplification, post-PCR cleanup, capture inline injection, and CE separation on the fully integrated microsystem were demonstrated using standard female 9947A genomic DNA. Figure 5.6 shows representative STR profiles obtained from 80 ng input standard DNA, demonstrating the feasibility of STR typing using this microsystem. The limit-of-detection testing using the standard DNA as well as analyses of blood stain samples is still underway to achieve results necessary for publication.

The total analysis time of this assay on the microsystem is about 2 hours and 35 min, which includes 40 min of DNA digestion and hybridization, 40 min of DNA template capture and washing, 45 min of PCR amplification, and 30 min of post-PCR cleanup, capture inline injection and CE. When directly analyzing forensic samples, such as blood stains, the processing time is estimated to be ~ 3 hours, because the cell lysis step takes 20 more minutes. Compared to conventional forensic STR typing (7-8 hours), at least 3-4 hours can be saved by using this system. Therefore, this microsystem can be utilized as an automated instrument for rapid forensic STR analysis in forensic laboratories. In addition, since the instrument for chip operation and detection has a small size, this fully integrated microsystem can be more effectively used as a portable system for on-site real-time human identification.

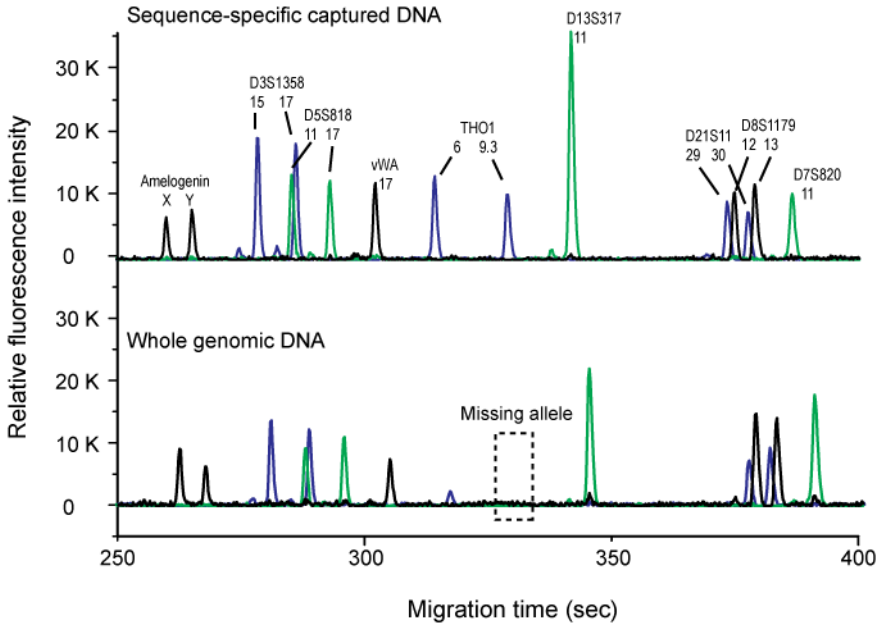

Figure 5.5. Comparison of the 9-plex STR profiles obtained from whole genomic DNA and purified DNA.

A total of 1 ng whole genomic DNA and ~1 ng DNA fragments purified from genomic DNA by sequence-specific DNA capture were amplified in a conventional PTC-200 thermocycler with a PCR protocol consisting of denaturation at 94 ^{o}C for 10 s, annealing at 58 ^{o}C for 20 s, and extension at 70 ^{o}C for 30 s. With these shortened heating times for PCR cycling, the DNA sample purified by sequence-specific DNA capture still provides full STR profiles. However, in the profiles from the whole genomic DNA, the TH01 9.3 allele drops out.

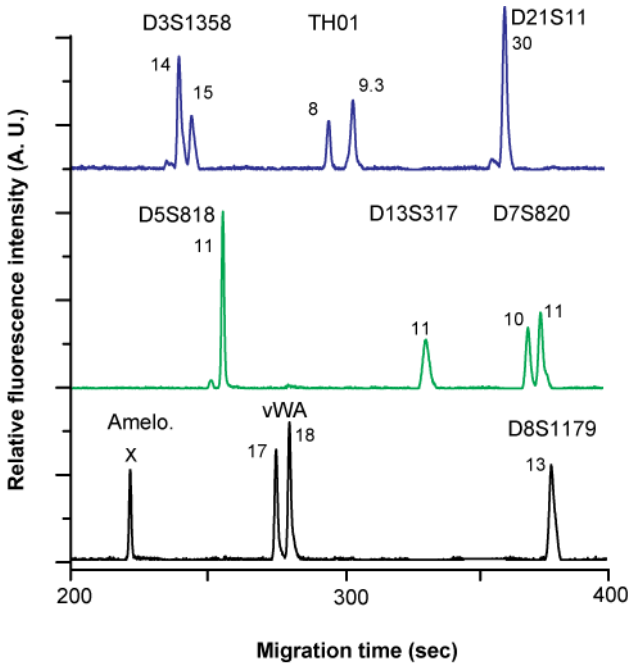

Figure 5.6. The 9-plex STR profiles obtained on the fully integrated microsystem.

With an 80-ng input of 9947A standard DNA, full profiles were successfully obtained using the microsystem. The timeline of the overall analysis is as follows: DNA digestion and hybridization: 40 min, DNA template capture and washing: 40 min, PCR amplification: 45 min, post-PCR cleanup, capture inline injection and CE: 30 min. The total analysis time is about 2 hours and 35 min. Compared to conventional forensic STR typing (7-8 hours) at least 3-4 hours can be saved by using this system and protocol.

5.5 Conclusion

A fully integrated microdevice capable of performing DNA extraction, PCR, post-PCR cleanup, and CE separation using a compact detection and control instrument and chip was successfully developed for rapid forensic STR analysis. Efficient sample transfer between each function units was achieved by the integration of sequence-specific DNA template capture using magnetic beads and post-PCR capture inline injection. The operation of this microdevice was optimized using standard DNA samples, validating the feasibility of rapid STR analysis on an integrated microfluidic system. This study is a significant step towards a fully integrated and portable forensic analysis system for rapid real-time human identification at crime scenes or other point-of-analysis situations.

Acknowledgements

We thank Susan Greenspoon at the Virginia Department of Forensic Science for technical discussion and advice. We also thank Promega Inc. for providing information about the PowerPlex® 16 STR system. The microdevice was microfabricated at the University of California, Berkeley Microfabrication Laboratory. We thank the UC Berkeley Chemistry Machine Shop for construction of the scanner system, and Henry Chan of the UC Berkeley Chemistry Electronic Shop for design and fabrication of the electronics. This project was supported by Grant No. 2007-DN-BX-K142 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice.

Chapter 6

Prospects

6.1 Summary

The technologies developed in previous chapters validate the feasibility of forensic DNA typing on an integrated microfluidic device to achieve rapid analysis with high sensitivity. To further advance the benefits provided by microfabrication technology, I propose the development of a 48-lane integrated microdevice which is capable of performing nano-liter PCR amplification, post-PCR purification, inline injection, and CE separation in a seamlessly integrated manner for high-throughput forensic STR analysis. Several engineering challenges in this high-throughput system need to be addressed, including the PCR heating system, microfluidic control, and the high-throughput UV exposure system for gel photopolymerization. The proposed 48-lane microsystem is also an excellent platform for exploring single-cell STR typing, which will positively impact the forensic STR analyses of mixture and low-copy-number DNA samples.

6.2 High-throughput PCR-capture-CE Microsystems

As the application of forensic DNA analysis has been extended to non-violent crimes such as property cases,[147] and as law enforcement agencies have become more sophisticated in the collection of evidence of probative value, the number of samples submitted for DNA examination has been increasing dramatically.[163] Although the integrated microfluidic system I have developed previously has demonstrated excellent performance for forensic STR analysis, including high speed, improved automation, and superb sensitivity, its throughput still cannot fully satisfy the requirements of current forensic investigations. In forensic casework analyses, positive and negative controls must be analyzed with evidence simultaneously to ensure a correct, contamination-free operation.[143] An automated system capable of analyzing multiple samples simultaneously is thus highly desired by the forensic community. Based on the previously developed microfluidic systems, I propose a 48-lane integrated microdevice consisting of PCR amplification, capture inline injection, and CE separation for high-throughput DNA typing. As illustrated in Figure 6.1, 48 identical PCR-capture-CE units are grouped into 24 doublets and radially arranged on a 6-inch glass wafer. Each doublet includes two PCR reactors with a microfabricated RTD and a external Peltier heater for thermal cycling, two capture gel inline injectors, two 14-cm-long CE channels with one shared cathode and one waste well. Although the basic operation this system has previously been tested in a single-lane format, several engineering challenges posed by this high-throughput microsystem must be carefully addressed.

6.2.1 Heating system design

STR amplification imposes a stringent requirement on the temperature accuracy across the entire PCR reactor because the multiplex amplification of STR loci should minimize false or non-specific amplicons. Although the microfabricated PCR heaters employed in my previous microsystems can provide extraordinary heating performance, several intrinsic drawbacks limit their integration with high-throughput systems. The microfabrication of these heaters requires multiple photolithography processes as well as several thin film metal processing steps, including sputtering, electroplating, and plasma etching. As a result, the total time and cost of microfabrication of the heaters could account for up to 70% of the whole microdevice, making it unaffordable. Furthermore, due to the large area covered by these fragile heaters in the multi-lane system, the manufacturing yield could be as low as 50%. Such large heaters often result in a higher risk of damage during operation as well as a higher complexity of training for new users.

To address this challenge, I propose to use external Peltier heaters for on-chip PCR thermal cycling. Peltier heaters have been extensively employed in conventional thermalcyclers and sub-microliter PCR microchip systems.[138, 164, 165] Since Peltier heaters can function as both heaters and coolers by simply reversing the direction of the applied voltage, the design of the thermal cycling system can be simplified in comparison with other heating methods. In this new heating system, 24 Peltier heaters are embedded into the stage of the scanner system, as shown in Figure 6.2. The position of the heaters is determined by the chip design. Flush contact between the microchip and the Peltier heaters is achieved by applying vacuum to the microchip. For temperature sensing, the same microfabricated RTD sensors as in the previous system are employed to provide accurate temperature measurement. This design will significantly lower the cost and time of microchip fabrication while providing adequate thermal cycling performance.

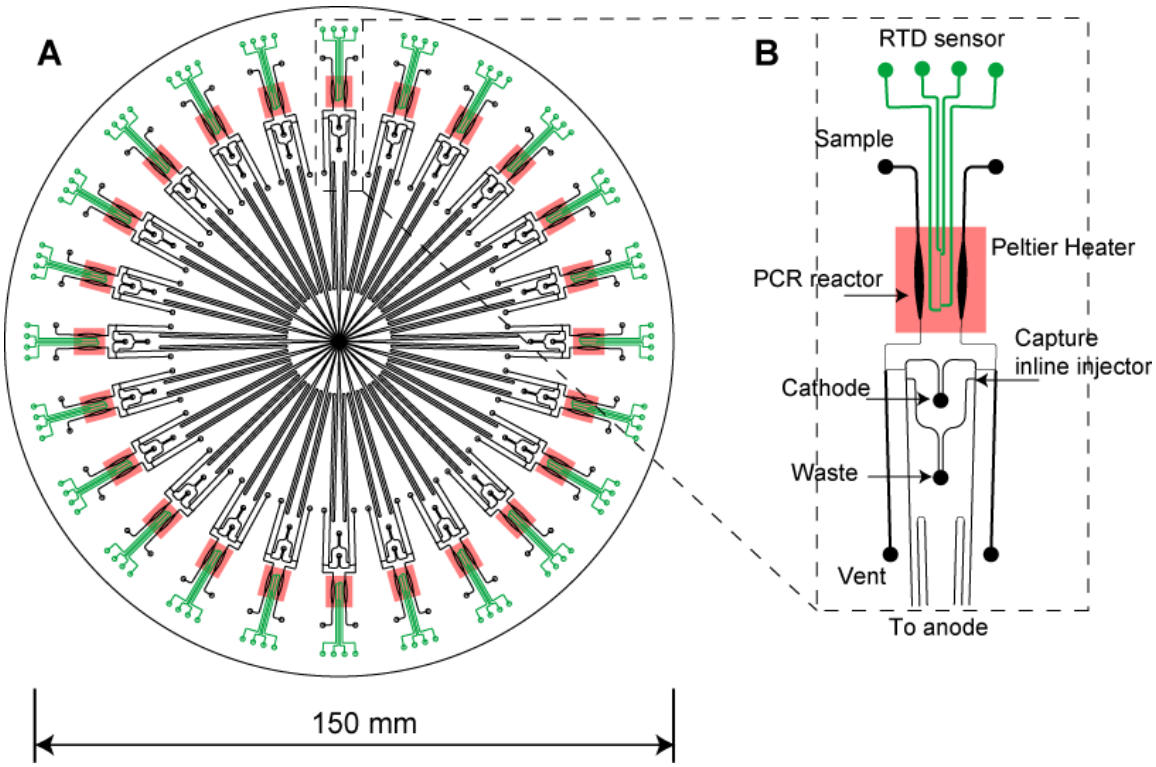

Figure 6.1. Schematic of the 48-lane PCR-capture-CE microdevice for forensic DNA Typing.

(A) A total of 48 lanes are radially arrayed on a 6" glass wafer. Each lane contains a PCR reactor, capture inline injector, and 14-cm-long CE channel. (B) The expanded view of the PCR reactors and capture inline injectors. Two independent reactors share one PCR heating system, one cathode and one waste reservoir.

6.2.2 Microfluidic control

The PDMS microvalves and micropumps developed by our group play essential roles for microfluidic control in integrated microsystems. In the previous PCR-CE microdevice, two microvalves were positioned on both sides of the PCR reactor to eliminate hydrodynamic flow during the thermal cycling. Though effective, these microvalves can be inconvenient during chip operation for several reasons. To avoid DNA carryover, the PDMS microvalves must be disassembled and the PDMS membranes have to be replaced for each run. This task makes the device hard to operate and time-consuming for high-throughput systems. In addition, microvalves may cause loading problems when dealing with cells or beads, because these small particles tend to get stuck in the via-holes of the valves. From the photopolymerization experiments, I have found that some viscous gels, such as 5% linear polyacrylamide (LPA) gel, can be used to effectively stop the hydrodynamatic flow in microchannels while still providing the electrical connections necessary for the subsequent CE separation. Therefore, in this 48-lane microsystem, PDMS microvalves can be eliminated and replaced by loading some stopping gels into the reservoirs prior to the thermal cycling. Considering the difficulty of loading the gel in an array chip, 3-mm-thick glass rings cut from a glass plate using a water jet cutter (AquaJet, Livermore, CA) can be thermally bonded to the microchip to function as reservoirs, as illustrated in Figure 6.2. Viscous gels can then be used to easily fill the glass grooves to stop the flow in all channels.

6.2.3 High-throughput exposure system

The capture inline injection developed in our group can provide near 100% injection efficiency for the electrophoretic separation, which is the key to achieving sensitive and reliable STR analysis on-chip. However, the photopolymerization of up to 48 gel plugs in a reasonably short time is a challenge. In my previous work, a Mercury lamp installed on a microscope was employed for gel photopolymerization through a photomask. Because this system can only expose one plug at a time with an exposure time of 5 min/plug, the waiting time for 48 gel plugs would be unreasonable. To resolve this problem, following conversation with Dr. James R. Scherer in the lab, I propose a low-cost UV exposure instrument for rapid photopolymerization of multiple capture gel plugs on-chip simultaneously. As shown in Figure 6.3, a bundle of optical fibers is employed to guide 365-nm UV light from a low-cost UV lamp to the photopolymerization spots on the microchip. The diameter of these fibers is carefully selected to match the length of capture gel plugs, so that no photomask is needed. Instead, the fiber holder is designed to match the features on the microdevice. The advantages of this instrument include high-speed exposure, low cost, and easy assembly. Preliminary work using a single fiber for photopolymerization has been completed and demonstrates very promising results.

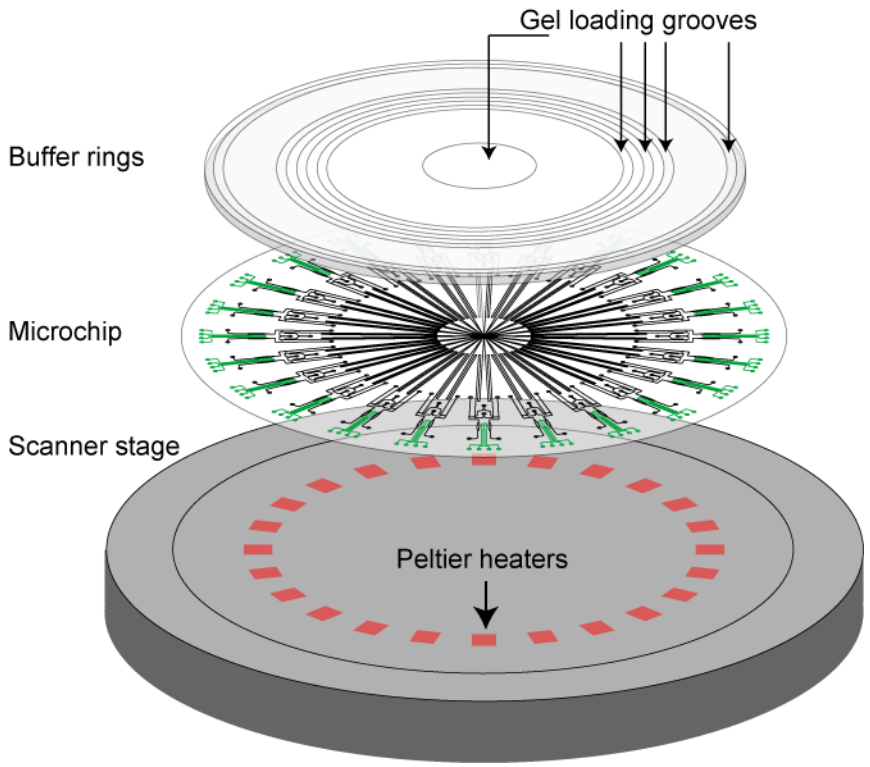

Figure 6.2. The exploded view of the microchip assembly.

Glass buffer rings cut by a water jet are thermally bonded with the microchip. During operation, the microchip is loaded onto the stage of the scanner. Vacuum is applied to hold the chip down and also to provide flush contact between the chip and the Peltier heaters embedded into the stage.

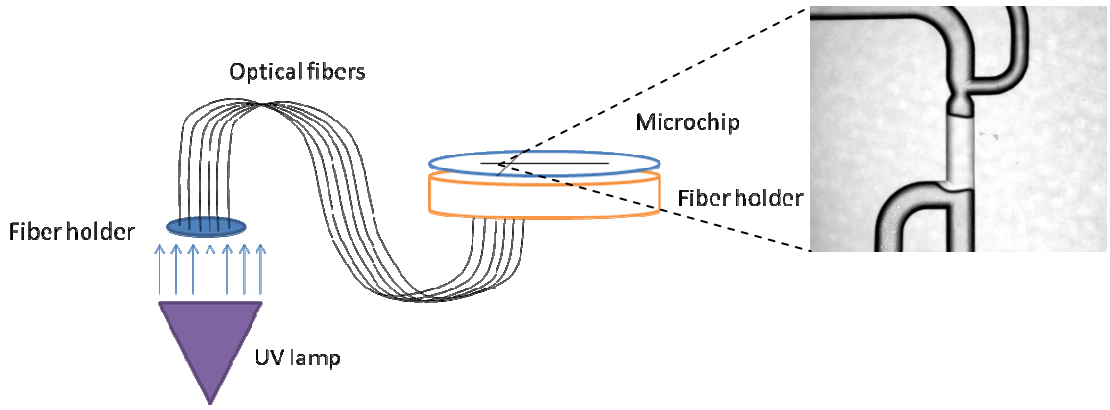

Figure 6.3. Schematic of the high-throughput UV exposure system.

Optical fibers are employed to guide UV light from a lamp to each photopolymerization spot on the chip. The embedded image shows a 400-µm-long capture gel plug obtained using this setup with a 500-µm-diameter optical fiber.

By using the method proposed above, the design and operation of this 48-lane PCR-capture-CE microdevice can be simplified significantly. Briefly, a photopolymer solution containing acrylamide monomers, streptavidin-acrylamide conjugates, and riboflavin, is first loaded into the CE channels, and then the stopping gel is added into the glass reservoir grooves to stop hydrodynamic flow in the channels. Using the high-throughput UV exposure system, 500-μm capture gel plugs are formed in all 48 lanes and the excess monomer solution is aspirated out. A separation matrix is then loaded from the central anode to the waste and from the cathode to the sample reservoirs to form a matrix-capture-matrix gel sandwich structure in the chip. In the next step, a well-mixed PCR cocktail is pipetted into each sample reservoir and drawn into the PCR chambers by applying vacuum on the vent reservoirs, followed by the loading of the stopping gels into the sample and vent reservoir grooves to seal the PCR chambers. After thermal cycling, the biotin-labeled PCR products from the PCR chambers are electrophoretically driven through the capture gel plug where they are bound efficiently via the biotin-streptavidin interaction. While DNA samples form a tightly concentrated plug, unbound materials are washed away in the following washing step. The fluorescently labeled DNA strands retained in the capture gel can be released into the separation channel for electrophoresis by heating the whole chip to 67 ^{o}C. The high performance provided by this integrated microdevice will enable some interesting research in the future that could help address some of the challenges of forensic investigations.

6.3 Application: Single-cell STR Typing

In forensic STR typing, researchers are facing unique challenges posed by samples that are mixtures from several contributors as well as low-copy-number (LCN) DNA samples from "touch evidence".[28, 29, 166] When dealing with mixture samples, DNA profiles consisting of all contributors are usually generated by a homogeneous PCR amplification. Expert systems are employed to extract the possible individual profiles based on ratios of peak height/area and statistic analysis, but as of yet, these systems have not been approved by the National DNA Index System (NDIS) for use with evidentiary samples.[30, 167] Excessive stutter peaks, allele drop-out/in, and stochastic effects associated with LCN samples may affect the accuracy of the interpretation. Laser microdissections[168] as well as other cell separation methods[169, 170] have been developed to separate samples completely prior to PCR, but the operation is slow and requires high precision to achieve pure separation.

In LCN DNA analysis (<100 pg or <33 copy template), many methods have been developed to enhance the PCR efficiency, including increasing the amplification cycle number[27], nested PCR[171], and whole-genome amplification[172], but they usually suffer from profile imbalance, allele drop-out/in, high stutter peaks, and sporadic and persistent contamination problems. One promising method is template concentration and reduced-volume PCR to improve the sensitivity.[173, 174] However, the minimum volume of 5 μL which can be performed in a tube has kept researchers from exploring smaller volumes. Post-PCR purification prior to the CE separation using spin columns has also proved useful for LCN sample analysis.[175] Although the signal intensities could be increased up to 4 fold, this extra step in forensic STR analysis is time consuming, increases the opportunity for sample mix-up and contamination, and cannot resolve stochastic effects associated with PCR, limiting its extensive application in forensic investigations. Improving the sensitivity of forensic STR typing to single-cell level will

not only benefit the LCN sample analysis, but also provide an ultimate way to resolve a mixture by generating a pure DNA profile from each individual cell present in a sample.

The 48-lane integrated microsystem with high throughput and high sensitivity proposed in the last section is an excellent platform for exploring single-cell STR typing. However, the first question will be "Is this microsystem sensitive enough to detect PCR products amplified from single genome copy?" Previously, our group developed a fully integrated microdevice which is capable of performing single-cell capture, reverse transcriptase-PCR, post-PCR product capture, inline injection and CE separation for gene expression analysis of single cells.[92] From this research, it is known that one single template can generate about 10^8 DNA fragments which are produced by 85 % amplification efficiency and 30 PCR cycles.[92] For STR multiplex amplifications, we can thus project that 10^6 - 10^7 DNA fragments will be generated for each allele in a single genome template. The limit-of-detection of our scanner system is ~20 pM of fluorescein dye samples[103], which is equal to ~$3x10^4$ molecules in a injection plug. Since the capture inline injection structure integrated in the 48-lane microdevice can provide similar CE injection efficiency to that in the microsystem for gene expression analysis of single cells, PCR products generated from a single template should be readily detectable using the 48-lane integrated microsystem.

In single-cell STR analysis, we are also concerned about stochastic effects, which are stochastically-induced allelic imbalance and drop-out of one or both alleles when only a few genomic DNA equivalents (<100 pg or 33 copies) are used to initiate PCR. The stochastic effects are likely induced by a combination of three factors: (i) Template loading variation. When adding low-concentration genomic DNA preparations into a PCR reaction, the actual copy numbers of each chromosome present in the tube conform to the Poisson distribution, resulting in the variation of PCR amplification on each allele. For example, if the expected copy number of template in a PCR reaction is 3 copies, then according to the Poisson distribution the probability that the copy numbers of all 30 chromosomes used in the PowerPlex® 16 kit will fall into the range of 1-5 copies is only 1.35 % (assuming each is introduced independently). Furthermore, the probability that at least one chromosome will not be present at all is 88.7 %. This statistical analysis reveals the fundamental cause of the stochastic variation of DNA profiles analyzed by conventional means. (ii) Accessibility of DNA template to primers. Due to chromosomal sequence and structural variations, the target region on each chromosome may provide different accessibility to corresponding primers, resulting in imbalanced STR profiles. Since the accessibility is highly related to template structures, it should be reproducible. (iii) Preferential amplification of alleles. This factor is not stochastic, but is well determined by the thermodynamics of allele sequence and length. Thus repeated typing of same samples should yield similar profiles. Problems ii and iii can thus be corrected or balanced by chemical means in the PCR reactions.

The "template loading variation" problem can be resolved by integrating a single-cell capture structure into the microchip. Single-cell loading followed by direct PCR amplification provides efficient sample transfer from a µL sample tube to a nL PCR reactor and guarantees that each allele has exactly the same starting template number (1 copy) for each allelic target. To enable single-cell capture on the chip, our group has successfully developed a cell capture method using DNA hybridization.[95, 176, 177] In this method, a size-limited 25×25-µm gold pad is microfabricated inside the PCR chamber for single-cell capture. By using a surface modification method described previously,[177] capture oligos (~20 bases) are deposited onto the gold pad and

the complementary oligos are attached to the cell surface. The procedure of covalently attaching ssDNA to cell surface is as follows: Thiolated single-stranded DNA is first exposed to 20 µL of the NHS-PEO_6-Maleimide solution at room temperature for 10 minutes. The reaction is purified by passing through a NAP-5 column that is pre-equilibrated with PBS solution (pH 7.2). The modified ssDNA is then incubated with suspensions of live cells in PBS at room temperature for 30 min, and then washed three times with PBS. Up to 120,000 DNA strands could be installed on each cell. Cells with ssDNA barcodes on their surface will be suspended in the PCR cocktail and drawn into all the PCR reactors by vacuum. When a cell flows over the gold cell capture pad, DNA hybridization occurs between the ssDNA on the cell and the gold pad. Since the size of gold pad is so small, only one cell can be captured and the uncaptured cells are washed out of the system using a well-mixed PCR cocktail without cells. In the 48-lane high-throughput microsystem, gold pads can be easily microfabricated in all the PCR chambers to facilitate single-cell capture.

Besides adjusting primer concentrations in a STR typing kit, the "accessibility" and "preferential amplification" factors can also be significantly controlled by operating the PCR in a nanoliter volume scale. Recent research[88, 178, 179] has demonstrated that PCR in pL-nL ranges could achieve a single-copy template detection limit due mainly to increased template concentrations and decreased diffusion distances in such small volumes.[52] Real-time PCR from single copy template in 10 pL droplets (10^6 smaller than conventional reaction volume) showed real-time PCR amplification curves with a cycle threshold (Ct) of ~18. This is 20 cycles earlier than that in commercial instruments.[180] This expected Ct shift of ~19.8 cycles (which compares with a 3.3-cycle threshold shift per 10-fold increase in starting copy concentration) indicates that decreasing the reaction volume has the same effect on PCR efficiency as increasing the template concentration. For example, by loading a single copy template in a 100 nL reactor, the PCR product concentration generated should be similar to that from 250 copies in a 25 µL reaction, which is already out of the LCN amplification range. This conclusion is also reasonable from the view of PCR kinetics.[181, 182] In a multiplex PCR reactions, the hybridization of primers and templates is the key step to ensure balanced amplifications. High template concentration produced by limited diffusion space favors the formation of primer-template complexes, which dramatically reduce the effects of preferential amplification. Thus, single-cell STR typing should be enhanced by performing PCR in nanoliter volume scale.

The proposed 48-lane microdevice with microfabricated gold pads in the PCR reactors will provide a reliable and robust platform for forensic STR typing of single cells due to its seamless integration of multiple analytical steps, automated operation process, and 100% efficient sample and product transport and analysis. This system has the potential to become the ultimate way for resolving mixture samples in forensic investigations, where intact cells can be recovered from evidence. For visually distinguishable cell mixtures, each different cell can be selectively loaded and typed on the device to get DNA profiles from each cell type. But for indistinguishable cell mixtures, we can repeatedly type randomly captured single cells to statistically obtain DNA profiles of all the cells and the ratio of cell types in the mixture.

Additionally, the enhanced process efficiency that is developed in this high-throughput PCR-cleanup-CE microdevice will dramatically enhance the performance of LCN and degraded DNA typing even when it is not in the stochastic single copy regime. Moreover, this fully integrated system could significantly reduce the time it takes to amplify and separate the DNA

samples and improve the reliability of these analyses, thus positively impacting the throughput needs of forensic laboratories.

6.4 Conclusions

Current forensic STR analysis is slow, requires significant technical expertise, and calls for the use of dedicated forensic laboratories. There is no doubt that DNA typing will be significantly improved by automation, miniaturization, and integration of the overall analytical process. Microfabrication technology possesses all the advantages which are needed to realize this goal. The integrated microsystems developed in this dissertation successfully address these issues by integrating novel techniques of sequence-specific DNA extraction and sample relocalization in PCR reactors with improved techniques of on-chip PCR-CE and post-PCR sample capture inline injection for multiplex forensic STR analysis. With these techniques, the integrated low-volume analysis will speed up the human identification process and lower the cost of investigation, helping to solve crimes and bring criminals to justice, as well as to free the wrongly accused in a timely manner. This work is a significant milestone towards a portable device that allows an individual with minimal training to perform highly sensitive, rapid DNA analyses in a setting outside of forensic laboratories.

References

1. Thompson, T., Black, S.M. Forensic Human Identification: An Introduction (CRC Press, Boca Raton, FL, 2007).
2. Faulds, H. On the Skin-Furrows of the Hand. *Nature* **22**, 605 (1880).
3. Gill, P., Jeffreys, A.J. & Werrett, D.J. Forensic Application of DNA Fingerprints. *Nature* **318**, 577-579 (1985).
4. Jeffreys, A.J., Wilson, V. & Thein, S.L. Individual-Specific Fingerprints of Human DNA. *Nature* **316**, 76-79 (1985).
5. Wambaugh, J. The Blooding (Bantam Books, New York, 1989).
6. Weir, B.S. DNA Statistics in the Simpson Matter. *Nature Genetics* **11**, 365-368 (1995).
7. Woodward, B. Shadow: Five Presidents and the Legacy of Watergate (Simon & Schuster, New York, 1999).
8. Nakamura, Y. et al. Variable Number of Tandem Repeat (Vntr) Markers for Human-Gene Mapping. *Science* **235**, 1616-1622 (1987).
9. Jobling, M.A., Gill, P. & Ep. Encoded evidence: DNA in forensic analysis. *Nature Reviews Genetics* **5**, 739-751 (2004).
10. Butler, J.M. Forensic STR typing: biology, technology and genetics of STR markers (Elsevier, New York, 2005).
11. Beckmann, J.S. & Weber, J.L. Survey of Human and Rat Microsatellites. *Genomics* **12**, 627-631 (1992).
12. Edwards, A., Civitello, A., Hammond, H.A. & Caskey, C.T. DNA Typing and Genetic-Mapping with Trimeric and Tetrameric Tandem Repeats. *American Journal of Human Genetics* **49**, 746-756 (1991).
13. CODIS -NDIS Statistics. *http://www.fbi.gov/hq/lab/html/codis1.htm*.
14. Butler, J.M. et al. Allele frequencies for 15 autosomal STIR loci on US caucasian, African American, and Hispanic populations. *Journal of Forensic Sciences* **48**, 908-911 (2003).
15. Butler, J.M. & Mv. Genetics and genomics of core short tandem repeat loci used in human identity testing. *Journal of Forensic Sciences* **51**, 253-265 (2006).
16. Kayser, M. et al. A comprehensive survey of human Y-chromosomal microsatellites. *American Journal of Human Genetics* **74**, 1183-1197 (2004).
17. Shewale, J.G., Sikka, S.C., Schneida, E., Sinha, S.K. & Pv. DNA profiling of azoospermic semen samples from vasectomized males by using Y-PLEX (TM) 6 amplification kit. *Journal of Forensic Sciences* **48**, 127-129 (2003).
18. Prinz, M., Ishii, A., Coleman, A., Baum, H.J. & Shaler, R.C. Validation and casework application of a Y chromosome specific STR multiplex. *Forensic Science International* **120**, 177-188 (2001).
19. Gill, P. et al. DNA Commission of the International Society of Forensic Genetics: recommendations on forensic analysis using Y-chromosome STRs. *International Journal of Legal Medicine* **114**, 305-309 (2001).
20. Brookes, A.J. The essence of SNPs. *Gene* **234**, 177-186 (1999).
21. Venter, J.C. et al. The sequence of the human genome. *Science* **291**, 1304-1351 (2001).
22. Sachidanandam, R. et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* **409**, 928-933 (2001).

23. Gill, P. An assessment of the utility of single nucleotide polymorphisms (SNPs) for forensic purposes. *International Journal of Legal Medicine* **114**, 204-210 (2001).
24. Wang, D.G. et al. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science* **280**, 1077-1082 (1998).
25. Allen, M. et al. Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: Sensitivity and matching probabilities. *Journal of Forensic Sciences* **43**, 453-464 (1998).
26. Andreasson, H., Asp, A., Alderborn, A., Gyllensten, U. & Allen, M. Mitochondrial sequence analysis for forensic identification using pyrosequencing technology. *Biotechniques* **32**, 124-+ (2002).
27. Gill, P. et al. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Science International* **112**, 17-40 (2000).
28. Butler, J.M., Shen, Y. & McCord, B.R. The development of reduced size STR amplicons as tools for analysis of degraded DNA. *Journal of Forensic Sciences* **48**, 1054-1064 (2003).
29. Wickenheiser, R.A. Trace DNA: A review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact. *Journal of Forensic Sciences* **47**, 442-450 (2002).
30. Gill, P. et al. Interpreting simple STR mixtures using allele peak areas. *Forensic Sci Int* **91**, 41-53 (1998).
31. Lee, H.C. & Ladd, C. Preservation and collection of biological evidence. *Croatian Medical Journal* **42**, 225-228 (2001).
32. Comey, C.T. et al. DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis. *Journal of Forensic Sciences* **39**, 1254-1269 (1994).
33. Walsh, P.S., Metzger, D.A. & Higuchi, R. Chelex-100 as a Medium for Simple Extraction of DNA for Pcr-Based Typing from Forensic Material. *Biotechniques* **10**, 506-513 (1991).
34. Greenspoon, S.A., Scarpetta, M.A., Drayton, M.L. & Turek, S.A. QIAamp spin columns as a method of DNA isolation for forensic casework. *Journal of Forensic Sciences* **43**, 1024-1030 (1998).
35. Greenspoon, S.A. et al. Application of the BioMek (R) 2000 laboratory automation workstation and the DNA IQ (TM) system to the extraction of forensic casework samples. *Journal of Forensic Sciences* **49**, 29-39 (2004).
36. Ng, L.K., Ng, A., Cholette, F. & Davis, C. Optimization of recovery of human DNA from envelope flaps using DNA IQ (TM) System for STR genotyping. *Forensic Science International-Genetics* **1**, 283-286 (2007).
37. Krenke, B.E. et al. Validation of a 16-locus fluorescent multiplex system. *Journal of Forensic Sciences* **47**, 773-785 (2002).
38. Promega Corporation, PowerPlex® 16 System. Technical Manual No. D012. . *Madison, WI* (2002).
39. Applied Biosystems. AmpFℓSTR® Profiler Plus® PCR Amplification Kit User's Manual. *Foster City, CA* (2000).
40. Butler, J.M., Buel, E., Crivellente, F., McCord, B.R. & Rk. Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. *Electrophoresis* **25**, 1397-1412 (2004).
41. Manz, A., Graber, N., Widmer, H.M. & Db. Miniaturized total chemical-analysis systems - a novel concept for chemical sensing. *Sensors and Actuators B-Chemical* **1**, 244-248 (1990).

42. Harrison, D.J. et al. Micromachining a Miniaturized Capillary Electrophoresis-Based Chemical-Analysis System on a Chip. *Science* **261**, 895-897 (1993).
43. Lander, E.S. et al. Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).
44. Wang, J. et al. The diploid genome sequence of an Asian individual. *Nature* **456**, 60-66 (2008).
45. Wheeler, D.A. et al. The complete genome of an individual by massively parallel DNA sequencing. *Nature* **452**, 872-877 (2008).
46. Check, E. Human genome: Patchwork people. *Nature* **437**, 1084-1086 (2005).
47. Ellegren, H. Comparative genomics and the study of evolution by natural selection. *Molecular Ecology* **17**, 4586-4596 (2008).
48. Acar, M., Mettetal, J.T. & van Oudenaarden, A. Stochastic switching as a survival strategy in fluctuating environments. *Nature Genetics* **40**, 471-475 (2008).
49. Choi, P.J., Cai, L., Frieda, K. & Xie, S. A stochastic single-molecule event triggers phenotype switching of a bacterial cell. *Science* **322**, 442-446 (2008).
50. Ivnitski, D. et al. Nucleic acid approaches for detection and identification of biological warfare and infectious disease agents. *Biotechniques* **35**, 862-869 (2003).
51. Rech, I. et al. Microchips and single-photon avalanche diodes for DNA separation with high sensitivity. *Electrophoresis* **27**, 3797-3804 (2006).
52. Jensen, K. Chemical kinetics - Smaller, faster chemistry. *Nature* **393**, 735-736 (1998).
53. Ferrance, J.P. et al. Developments toward a complete micro-total analysis system for Duchenne muscular dystrophy diagnosis. *Analytica Chimica Acta* **500**, 223-236 (2003).
54. Blazej, R.G., Kumaresan, P., Mathies, R.A. & Gt. Microfabricated bioprocessor for integrated nanoliter-scale Sanger DNA sequencing. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 7240-7245 (2006).
55. Panaro, N.J. et al. Evaluation of DNA fragment sizing and quantification by the Agilent 2100 Bioanalyzer. *Clinical Chemistry* **46**, 1851-1853 (2000).
56. Quake, S.R. & Scherer, A. From micro- to nanofabrication with soft materials. *Science* **290**, 1536-1540 (2000).
57. Fiorini, G.S. & Chiu, D.T. Disposable microfluidic devices: fabrication, function, and application. *Biotechniques* **38**, 429-446 (2005).
58. Lagally, E.T., Emrich, C.A., Mathies, R.A. & Un. Fully integrated PCR-capillary electrophoresis microsystem for DNA analysis. *Lab on a Chip* **1**, 102-107 (2001).
59. Burns, M.A. et al. Microfabricated structures for integrated DNA analysis. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 5556-5561 (1996).
60. Fukuba, T., Yamamoto, T., Naganuma, T. & Fujii, T. Microfabricated flow-through device for DNA amplification - towards in situ gene analysis. *Chemical Engineering Journal* **101**, 151-156 (2004).
61. Huhmer, A.F.R. & Landers, J.P. Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes. *Analytical Chemistry* **72**, 5507-5512 (2000).
62. Luharuka, R. & Hesketh, P.J. A bistable electromagnetically actuated rotary gate microvalve. *Journal of Micromechanics and Microengineering* **18**, - (2008).
63. Li, H.Q. et al. Fabrication of a high frequency piezoelectric microvalve. *Sensors and Actuators a-Physical* **111**, 51-56 (2004).

64. Lagally, E.T. et al. Integrated portable genetic analysis microsystem for pathogen/infectious disease detection. *Analytical Chemistry* **76**, 3162-3170 (2004).
65. Marcus, J.S., Anderson, W.F. & Quake, S.R. Microfluidic single-cell mRNA isolation and analysis. *Analytical Chemistry* **78**, 3084-3089 (2006).
66. Beebe, D.J. et al. Functional hydrogel structures for autonomous flow control inside microfluidic channels. *Nature* **404**, 588-590 (2000).
67. Yu, C. et al. Flow control valves for analytical microfluidic chips without mechanical parts based on thermally responsive monolithic polymers. *Analytical Chemistry* **75**, 1958-1961 (2003).
68. Liu, R.H. et al. Validation of a fully integrated microfluidic array device for influenza A subtype identification and sequencing. *Analytical Chemistry* **78**, 4184-4193 (2006).
69. Zhao, B., Viernes, N.O.L., Moore, J.S. & Beebe, D.J. Control and applications of immiscible liquids in microchannels. *Journal of the American Chemical Society* **124**, 5284-5285 (2002).
70. Lee, S.H., Cho, S.I., Lee, C.S., Kim, B.G. & Kim, Y.K. Microfluidic chip for biochemical reaction and electrophoretic separation by quantitative volume control. *Sensors and Actuators B-Chemical* **110**, 164-173 (2005).
71. Koh, C.G. et al. Integrating polymerase chain reaction, valving, and electrophoresis in a plastic device for bacterial detection. *Analytical Chemistry* **75**, 4591-4598 (2003).
72. Easley, C.J. et al. A fully integrated microfluidic genetic analysis system with sample-in-answer-out capability. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 19272-19277 (2006).
73. Liu, C.N., Toriello, N.M., Mathies, R.A. & Rw. Multichannel PCR-CE microdevice for genetic analysis. *Analytical Chemistry* **78**, 5474-5479 (2006).
74. Ueberfeld, J., El-Difrawy, S.A., Ramdhanie, K. & Ehrlich, D.J. Solid-support sample loading for DNA sequencing. *Analytical Chemistry* **78**, 3632-3637 (2006).
75. Beyor, N., Seo, T.S., Liu, P. & Mathies, R.A. Immunomagnetic bead-based cell concentration microdevice for dilute pathogen detection. *Biomedical Microdevices* **10**, 909-917 (2008).
76. Khandurina, J., Jacobson, S.C., Waters, L.C., Foote, R.S. & Ramsey, J.M. Microfabricated porous membrane structure for sample concentration and electrophoretic analysis. *Analytical Chemistry* **71**, 1815-1819 (1999).
77. Blazej, R.G., Kumaresan, P., Cronier, S.A., Mathies, R.A. & Jk. Inline injection microdevice for attomole-scale sanger DNA sequencing. *Analytical Chemistry* **79**, 4499-4506 (2007).
78. Long, Z.C., Shen, Z., Wu, D.P., Qin, J.H. & Lin, B.C. Integrated multilayer microfluidic device with a nanoporous membrane interconnect for online coupling of solid-phase extraction to microchip electrophoresis. *Lab on a Chip* **7**, 1819-1824 (2007).
79. Woolley, A.T. et al. Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device. *Analytical Chemistry* **68**, 4081-4086 (1996).
80. Thaitrong, N., Toriello, N.M., Del Bueno, N. & Mathies, R.A. Polymerase Chain Reaction-Capillary Electrophoresis Genetic Analysis Microdevice with In-Line Affinity Capture Sample Injection. *Analytical Chemistry* **81**, 1371-1377 (2009).

81. Yeung, S.H.I., Liu, P., Del Bueno, N., Greenspoon, S.A. & Mathies, R.A. Integrated Sample Cleanup-Capillary Electrophoresis Microchip for High-Performance Short Tandem Repeat Genetic Analysis. *Anal. Chem.* **81**, 210-217 (2009).
82. Levene, M.J. et al. Zero-mode waveguides for single-molecule analysis at high concentrations. *Science* **299**, 682-686 (2003).
83. Korlach, J. et al. Selective aluminum passivation for targeted immobilization of single DNA polymerase molecules in zero-mode waveguide nanostructures. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 1176-1181 (2008).
84. Emrich, C.A., Tian, H.J., Medintz, I.L., Mathies, R.A. & Xy. Microfabricated 384-lane capillary array electrophoresis bioanalyzer for ultrahigh-throughput genetic analysis. *Analytical Chemistry* **74**, 5076-5083 (2002).
85. Paegel, B.M. et al. High throughput DNA sequencing with a microfabricated 96-lane capillary array electrophoresis bioprocessor. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 574-579 (2002).
86. Fredlake, C.P. et al. Ultrafast DNA sequencing on a microchip by a hybrid separation mechanism that gives 600 bases in 6.5 minutes. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 476-481 (2008).
87. Aborn, J.H. et al. A 768-lane microfabricated system for high-throughput DNA sequencing. *Lab on a Chip* **5**, 669-674 (2005).
88. Kumaresan, P., Yang, C.J., Cronier, S.A., Blazei, R.G. & Mathies, R.A. High-throughput single copy DNA amplification and cell analysis in engineered nanoliter droplets. *Analytical Chemistry* **80**, 3522-3529 (2008).
89. Cai, L., Friedman, N. & Xie, X.S. Stochastic protein expression in individual cells at the single molecule level. *Nature* **440**, 358-362 (2006).
90. Eilken, H.M., Nishikawa, S.I. & Schroeder, T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* **457**, 896-U159 (2008).
91. Quintana, E. et al. Efficient tumour formation by single human melanoma cells. *Nature* **456**, 593-U33 (2008).
92. Toriello, N.M. et al. Integrated Microfluidic Bioprocessor for Single-Cell Gene Expression Analysis. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 20173-20178 (2008).
93. Zhong, J.F. et al. A microfluidic processor for gene expression profiling of single human embryonic stem cells. *Lab on a Chip* **8**, 68-74 (2008).
94. Bontoux, N. et al. Integrating whole transcriptome assays on a lab-on-a-chip for single cell gene profiling. *Lab on a Chip* **8**, 443-450 (2008).
95. Douglas, E.S., Chandra, R.A., Bertozzi, C.R., Mathies, R.A. & Francis, M.B. Self-assembled cellular microarrays patterned using DNA barcodes. *Lab on a Chip* **7**, 1442-1448 (2007).
96. Toriello, N.M., Liu, C.N., Mathies, R.A. & Tz. Multichannel reverse transcription-polymerase chain reaction microdevice for rapid gene expression and biomarker analysis. *Analytical Chemistry* **78**, 7997-8003 (2006).
97. Toriello, N.M., Liu, C.N., Blazej, R.G., Thaitrong, N. & Mathies, R.A. Integrated affinity capture, purification, and capillary electrophoresis microdevice for quantitative double-stranded DNA analysis. *Analytical Chemistry* **79**, 8549-8556 (2007).

98. Kaigala, G.V. et al. Automated screening using microfluidic chip-based PCR and product detection to assess risk of BK virus-associated nephropathy in renal transplant recipients. *Electrophoresis* **27**, 3753-3763 (2006).
99. Prakash, A.R., De La Rosa, C., Fox, J.D. & Kaler, K.V.I.S. Identification of respiratory pathogen Bordetella Pertussis using integrated microfluidic chip technology. *Microfluidics and Nanofluidics* **4**, 451-456 (2008).
100. Pal, R. et al. An integrated microfluidic device for influenza and other genetic analyses. *Lab on a Chip* **5**, 1024-1032 (2005).
101. Beyor, N., Yi, L., Seo, T.S. & Mathies, R.A. Integrated Capture, Concentration, PCR, and Capillary Electrophoretic Analysis of Pathogens on a Chip. *Analytical Chemistry* **(Accepted)** (2009).
102. Schmalzing, D. et al. DNA typing in thirty seconds with a microfabricated device. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10273-10278 (1997).
103. Yeung, S.H.I. et al. Rapid and high-throughput forensic short tandem repeat typing using a 96-lane microfabricated capillary array electrophoresis microdevice. *Journal of Forensic Sciences* **51**, 740-747 (2006).
104. Greenspoon, S.A. et al. A forensic laboratory tests the Berkeley microfabricated capillary array electrophoresis device. *Journal of Forensic Sciences* **53**, 828-837 (2008).
105. Liu, P. et al. Integrated portable polymerase chain reaction-capillary electrophoresis microsystem for rapid forensic short tandem repeat typing. *Analytical Chemistry* **79**, 1881-1889 (2007).
106. Liu, P. et al. Real-time forensic DNA analysis at a crime scene using a portable microchip analyzer. *Forensic Sci. Intl.: Genetics* **2**, 301-309 (2008).
107. Edwards, A., Civitello, A., Hammond, H.A., Caskey, C.T. & Gg. DNA TYPING AND GENETIC-MAPPING WITH TRIMERIC AND TETRAMERIC TANDEM REPEATS. *American Journal of Human Genetics* **49**, 746-756 (1991).
108. Chakraborty, R. et al. The utility of short tandem repeat loci beyond human identification: Implications for development of new DNA typing systems. *Electrophoresis* **20**, 1682-1696 (1999).
109. deMello, A.J. & Ci. Control and detection of chemical reactions in microfluidic systems. *Nature* **442**, 394-402 (2006).
110. Dittrich, P.S., Tachikawa, K., Manz, A. & Rz. Micro total analysis systems. Latest advancements and trends. *Analytical Chemistry* **78**, 3887-3907 (2006).
111. Tian, H.J., Huhmer, A.F.R., Landers, J.P. & Kb. Evaluation of silica resins for direct and efficient extraction of DNA from complex biological matrices in a miniaturized format. *Analytical Biochemistry* **283**, 175-191 (2000).
112. Breadmore, M.C. et al. Microchip-based purification of DNA from biological samples. *Analytical Chemistry* **75**, 1880-1886 (2003).
113. Bienvenue, J.M. et al. Microchip-based cell lysis and DNA extraction from sperm cells for application to forensic analysis. *Journal of Forensic Sciences* **51**, 266-273 (2006).
114. Wilding, P., Shoffner, M.A., Kricka, L.J. & Pe. PCR IN A SILICON MICROSTRUCTURE. *Clinical Chemistry* **40**, 1815-1818 (1994).
115. Kopp, M.U., de Mello, A.J., Manz, A. & Zn. Chemical amplification: Continuous-flow PCR on a chip. *Science* **280**, 1046-1048 (1998).

116. Medintz, I. et al. High-performance multiplex SNP analysis of three hemochromatosis-related mutations with capillary array electrophoresis microplates. *Genome Research* **11**, 413-421 (2001).
117. Roper, M.G., Easley, C.J., Landers, J.P. & Hv. Advances in polymerase chain reaction on microfluidic chips. *Analytical Chemistry* **77**, 3887-3893 (2005).
118. Zhang, C.S., Xu, J.L., Ma, W.L., Zheng, W.L. & Wo. PCR microfluidic devices for DNA amplification. *Biotechnology Advances* **24**, 243-284 (2006).
119. Lagally, E.T., Simpson, P.C., Mathies, R.A. & Nt. Monolithic integrated microfluidic DNA amplification and capillary electrophoresis analysis system. *Sensors and Actuators B-Chemical* **63**, 138-146 (2000).
120. Lagally, E.T., Medintz, I., Mathies, R.A. & Wv. Single-molecule DNA amplification and analysis in an integrated microfluidic device. *Analytical Chemistry* **73**, 565-570 (2001).
121. U. S. Department of Justice, Federal Bureau of Investigation. Crime in the United States. *http://www.fbi.gov/ucr/05cius/index.html*. (2005).
122. Grover, W.H. et al. Monolithic membrane valves and diaphragm pumps for practical large-scale integration into glass microfluidic devices. *Sensors and Actuators B-Chemical* **89**, 315-323 (2003).
123. Medintz, I.L. et al. Genotyping energy-transfer-cassette-labeled short-tandem-repeat amplicons with capillary array electrophoresis microchannel plates. *Clinical Chemistry* **47**, 1614-1621 (2001).
124. Sullivan, K.M., Mannucci, A., Kimpton, C.P. & Gill, P. A Rapid and Quantitative DNA Sex Test - Fluorescence-Based Pcr Analysis of X-Y Homologous Gene Amelogenin. *Biotechniques* **15**, 636-& (1993).
125. Wilson, I.G. & Xy. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63**, 3741-3751 (1997).
126. Radstrom, P. et al. Pre-PCR processing - Strategies to generate PCR-compatible samples. *Molecular Biotechnology* **26**, 133-146 (2004).
127. Bar, W., Kratzer, A., Machler, M. & Schmid, W. POSTMORTEM STABILITY OF DNA. *Forensic Science International* **39**, 59-70 (1988).
128. Burger, M.F., Song, E.Y., Schumm, J.W. & Ar. Buccal DNA samples for DNA typing: new collection and processing methods. *Biotechniques* **39**, 257-261 (2005).
129. Opel, K.L. et al. The application of miniplex primer sets in the analysis of degraded DNA from human skeletal remains. *Journal of Forensic Sciences* **51**, 351-356 (2006).
130. Primorac, D., Schanfield, M.S. & Cq. Application of forensic DNA testing in the legal system. *Croatian Medical Journal* **41**, 32-46 (2000).
131. Belgrader, P., Smith, J.K., Weedn, V.W., Northrup, M.A. & Ze. Rapid PCR for identity testing using a battery-powered miniature thermal cycler. *Journal of Forensic Sciences* **43**, 315-319 (1998).
132. Andersen, J.F. et al. Further validation of a multiplex STR system for use in routine forensic identity testing. *Forensic Science International* **78**, 47-64 (1996).
133. Medintz, I.L. et al. High-performance genetic analysis using microfabricated capillary array electrophoresis microplates. *Electrophoresis* **22**, 3845-3856 (2001).
134. Horsman, K.M., Bienvenue, J.M., Blasier, K.R., Landers, J.P. & No. Forensic DNA analysis on microfluidic devices: A review. *Journal of Forensic Sciences* **52**, 784-799 (2007).

135. Mennell, J., Shaw, I. & Mx. The future of forensic and crime scene science - Part I. A UK forensic science user and provider perspective. *Forensic Science International* **157**, S7-S12 (2006).
136. Schmidt, U. et al. Low-volume amplification on chemically structured chips using the PowerPlex16 DNA amplification kit. *International Journal of Legal Medicine* **120**, 42-48 (2006).
137. Goedecke, N. et al. A high-performance multilane microdevice system designed for the DNA forensics laboratory. *Electrophoresis* **25**, 1678-1686 (2004).
138. Zhou, Z.M. et al. Determination of SARS-coronavirus by a microfluidic chip system. *Electrophoresis* **25**, 3032-3039 (2004).
139. Dunn, W.C. et al. PCR amplification and analysis of simple sequence length polymorphisms in mouse DNA using a single microchip device. *Analytical Biochemistry* **277**, 157-160 (2000).
140. Burns, M.A. et al. An integrated nanoliter DNA analysis device. *Science* **282**, 484-487 (1998).
141. Waters, L.C. et al. Multiple sample PCR amplification and electrophoretic analysis on a microchip. *Analytical Chemistry* **70**, 5172-5176 (1998).
142. Greenspoon, S.A. et al. Validation and implementation of the PowerPlex (R) 16 BIO system STR multiplex for forensic casework. *Journal of Forensic Sciences* **49**, 71-80 (2004).
143. DNA Advisory Board, Quality assurance standards for forensic DNA testing laboratories and for convicted offender DNA databasing laboratories. *Forensic Sci. Commun.* *http://www.fbi.gov/hq/lab/fsc/backissu/july2000/codispre.htm* **2** (2000).
144. Bond, J.W. & Ea. Value of DNA evidence in detecting crime. *Journal of Forensic Sciences* **52**, 128-136 (2007).
145. Bureau of Justice Statistics, U.S. Department of Justice, Office of Justice Programs, Reentry trends in the United States. *http://www.ojp.usdoj.gov/bjs/*.
146. Paegel, B.M., Yeung, S.H.I. & Mathies, R.A. Microchip bioprocessor for integrated nanovolume sample purification and DNA sequencing. *Analytical Chemistry* **74**, 5092-5098 (2002).
147. National Institute of Justice. NIJ 2007 annual report. *http://www.ojp.usdoj.gov/nij/about/annual-reports.htm*.
148. Liu, P. & Mathies, R.A. Integrated microfluidic systems for high-performance genetic analysis. *Trends in Biotechnology* **27**, 572-581 (2009).
149. Wen, J., Guillo, C., Ferrance, J.P. & Landers, J.P. Microfluidic-based DNA purification in a two-stage, dual-phase microchip containing a reversed-phase and a photopolymerized monolith. *Analytical Chemistry* **79**, 6135-6142 (2007).
150. Giese, H., Lam, R., Selden, R. & Tan, E. Fast Multiplexed Polymerase Chain Reaction for Conventional and Microfluidic Short Tandem Repeat Analysis. *Journal of Forensic Sciences* **54**, 1287-1296 (2009).
151. Refseth, U.H., Fangan, B.M. & Jakobsen, K.S. Hybridization capture of microsatellites directly from genomic DNA. *Electrophoresis* **18**, 1519-1523 (1997).
152. Archer, M.J., Lin, B.C., Wang, Z. & Stenger, D.A. Magnetic bead-based solid phase for selective extraction of genomic DNA. *Analytical Biochemistry* **355**, 285-297 (2006).
153. Legler, T.J. et al. Specific magnetic bead-based capture of free fetal DNA from maternal plasma. *Transfusion and Apheresis Science* **40**, 153-157 (2009).

154. Yeung, S.W. & Hsing, I.M. Manipulation and extraction of genomic DNA from cell lysate by functionalized magnetic particles for lab on a chip applications. *Biosensors & Bioelectronics* **21**, 989-997 (2006).
155. Parham, N.J. et al. Specific magnetic bead-based capture of genomic DNA from clinical samples: Application to the detection of group B streptococci in Vaginal/Anal swabs. *Clinical Chemistry* **53**, 1570-1576 (2007).
156. Shi, Y.N. et al. Radial capillary array electrophoresis microplate and scanner for high-performance nucleic acid analysis. *Analytical Chemistry* **71**, 5354-5361 (1999).
157. Oefner, P.J. et al. Efficient random subcloning of DNA sheared in a recirculating point-sink flow system. *Nucleic Acids Research* **24**, 3879-3886 (1996).
158. Joneja, A. & Huang, X.H. A device for automated hydrodynamic shearing of genomic DNA. *Biotechniques* **46**, 553-+ (2009).
159. Hengen, P.N. Methods and reagents - Shearing DNA for genomic library construction. *Trends in Biochemical Sciences* **22**, 273-274 (1997).
160. Deininger, P.L. Approaches to Rapid DNA-Sequence Analysis. *Analytical Biochemistry* **135**, 247-263 (1983).
161. Andersson, B. et al. Adapter-Based Uracil DNA Glycosylase Cloning Simplifies Shotgun Library Construction for Large-Scale Sequencing. *Analytical Biochemistry* **218**, 300-308 (1994).
162. Osoegawa, K. et al. A bacterial artificial chromosome library for sequencing the complete human genome. *Genome Research* **11**, 483-496 (2001).
163. National Institute of Justice. NIJ 2006 annual report. *http://www.ojp.usdoj.gov/nij/about/annual-reports.htm*.
164. Khandurina, J. et al. Integrated system for rapid PCR-based DNA analysis in microfluidic devices. *Analytical Chemistry* **72**, 2995-3000 (2000).
165. Liu, Y.J. et al. DNA amplification and hybridization assays in integrated plastic monolithic devices. *Analytical Chemistry* **74**, 3063-3070 (2002).
166. Whitaker, J.P., Cotton, E.A. & Gill, P. A comparison of the characteristics of profiles produced with the AMPFlSTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis. *Forensic Sci Int* **123**, 215-23 (2001).
167. Clayton, T.M., Whitaker, J.P., Sparkes, R. & Gill, P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. *Forensic Sci Int* **91**, 55-70 (1998).
168. Sanders, C.T., Sanchez, N., Ballantyne, J. & Peterson, D.A. Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. *J. Forensic Sci.* **51**, 748-757 (2006).
169. Schoell, W.M.J., Klintschar, M., Mirhashemi, R. & Pertl, B. Separation of sperm and vaginal cells with flow cytometry for DNA typing after sexual assault. *Obstetrics and Gynecology* **94**, 623-627 (1999).
170. Horsman, K.M. et al. Separation of sperm and epithelial cells in a microfabricated device: Potential application to forensic analysis of sexual assault evidence. *Analytical Chemistry* **77**, 742-749 (2005).
171. Strom, C.M. & Rechitsky, S. Use of nested PCR to identify charred human remains and minute amounts of blood. *Journal of Forensic Sciences* **43**, 696-700 (1998).
172. Hanson, E.K. & Ballantyne, J. Whole genome amplification strategy for forensic genetic analysis using single or few cell equivalents of genomic DNA. *Analytical Biochemistry* **346**, 246-257 (2005).

173. Leclair, B. et al. STR DNA typing: Increased sensitivity and efficient sample consumption using reduced PCR reaction volumes. *Journal of Forensic Sciences* **48**, 1001-1013 (2003).
174. Gaines, M.L., Wojtkiewicz, P.W., Valentine, J.A. & Brown, C.L. Reduced volume PCR amplification reactions using the AmpF rho STR (R) profiler plus (TM) kit. *J. Forensic Sci.* **47**, 1224-1237 (2002).
175. Smith, P.J. & Ballantyne, J. Simplified low-copy-number DNA analysis by Post-PCR purification. *Journal of Forensic Sciences* **52**, 820-829 (2007).
176. Toriello, N.M., Douglas, E.S. & Mathies, R.A. Microfluidic device for electric field-driven single-cell capture and activation. *Analytical Chemistry* **77**, 6935-6941 (2005).
177. Hsiao, S.C. et al. Direct Cell Surface Modification with DNA for the Capture of Primary Cells and the Investigation of Myotube Formation on Defined Patterns. **(In preparation)** (2008).
178. Diehl, F. et al. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nature Methods* **3**, 551-559 (2006).
179. Ottesen, E.A., Hong, J.W., Quake, S.R. & Leadbetter, J.R. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science* **314**, 1464-1467 (2006).
180. Beer, N.R. et al. On-chip, real-time, single-copy polymerase chain reaction in picoliter droplets. *Analytical Chemistry* **79**, 8471-8475 (2007).
181. Gevertz, J.L., Dunn, S.M. & Roth, C.M. Mathematical model of real-time PCR kinetics. *Biotechnology and Bioengineering* **92**, 346-355 (2005).
182. Mehra, S. & Hu, W.S. A kinetic model of quantitative real-time polymerase chain reaction. *Biotechnology and Bioengineering* **91**, 848-860 (2005).