Microfluidic Technology for High-Throughput Single Cell Gene Expression Analysis

by

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Abstract

Transcription measurements with single cell resolution are critical to understanding variable responses in immunity, measuring stochastic noise in gene expression, and assessing the disease and developmental state of heterogeneous populations. The latter is particularly important in stem cell science, developmental biology, and cancer, where minority cells may be most significant. To see these populations requires the quick and cost-effective measurement of hundreds to thousands of individual cells. Quantitative real-time polymerase chain reaction (RT-qPCR) is a sensitive method for quantitative analysis of transcript levels that provides excellent sensitivity and dynamic range in the detection of transcripts. However, the use of RT-qPCR is generally limited to ensemble measurements of bulk cells or plasma, and is blind to minority cell populations. This aggregation obscures the underlying biological response and variability. To address this limitation, we exploit recent advances in scalable microfluidics to develop robust lab-on-chip technology capable of highly parallel and cost-effective measurements of transcript levels from single cells. The microfluidic device integrates single-cell capture, lysis, reverse transcription of contained RNA, and precise measurement of cDNA using RT-qPCR. We demonstrate this system in the study of microRNA expression in a cell line representing chronic myelogenous leukemia, pluripotency markers in differentiating human embryonic stem cells, and the detection of somatic mutations in a primary breast cancer sample. The ability to screen isolated cells by simultaneously measuring the fraction of cells expressing a specific gene and quantifying the abundance of expression, may provide a new modality for the early detection of disease such as cancer.
Preface

The work presented in this thesis is part of a collaborative effort to develop and apply microfluidic systems for single cell genomics. A manuscript entitled *High-Throughput Microfluidic RT-qPCR of Single Cells* by Adam White, Michael VanInsberghe, Oleh Petriv, Mani Hamidi, Darek Sikorski, Marco A. Marra, James M. Piret, Sam Aparicio, and Carl L. Hansen has been submitted for publication at Nature Methods and is currently under review. The content of this manuscript is presented in Chapter 2. Specifically, the collaborators contributed the following: AW and MV designed and fabricated microfluidic devices. AW, MV, OP, and MH performed on-chip experiments and analyzed data. MV developed image analysis code. OP and MH developed Oct4 assays and performed off-chip experiments. DS performed hESC differentiation cultures and mRNA FISH measurements. CH, SA, MM, and JP designed research. AW and CH wrote the manuscript.
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Chapter 1

Introduction

1.1 Why Measure Gene Expression?

The cells of all organisms inherit genetic instructions that govern their behaviour. This complete set of genetic instructions (the genome) is composed of DNA and contains the code for genes. Cell differences arise as a result of different genes being actively expressed (or not). Gene expression is the process by which genetic information is transcribed from DNA into RNA, and this RNA is translated into proteins (or processed into bioactive non-coding RNA). These proteins are the chief components of the complicated molecular interactions inside a cell, performing tasks including catalysis of chemical reactions, intra- and extra-cellular signaling, and maintaining structural integrity. In order to maintain life, the type of proteins, abundance, and timing of gene expression is tightly regulated. A single gene can have profoundly different functions depending on the timing, location, and amount of gene expression [1]. Gene expression is regulated at the levels of transcription, RNA processing, translation, post-translational modifications, and degradation, allowing cells to respond to their environment. The transcriptome, encompassing all RNA transcripts of the cell, represents all proteins that are actively being synthesized and thus provides a unique signature of cell state. Thus, measuring transcripts allows for directly studying cellular processes and variation.

1.2 Transcriptional Variability Between Single Cells

Much of our biomolecular knowledge of cells and cell tissue is the result of gene expression measurements of transcription. However, transcription measurements are traditionally performed on bulk samples of large numbers (thousands to millions) of cells. The transcriptional variability between individual cells is obscured by ensemble
averaging. Heterogeneity is an ever present feature of biological systems, and cellular heterogeneity has been observed in cell types ranging from bacterial cells to mammalian cells. The sources of transcriptional variability between single cells include the stochastic nature of transcription, different stages of cell cycle, differentiation, and disease.

Although heterogeneity between two individual cells often arises through differences in the genome, even isogenetic cells may exhibit large variability in transcript expression. These differences may occur through several different mechanisms. The molecular kinetics involved in gene expression make it a stochastic process, subject to noise [2, 3]. This results in bursts of expression and apparently random fluctuations that contribute to phenotypic variation through various feedback mechanisms [2, 3].

Gene expression will also naturally vary between cells in different stages of the cell division cycle. Cell division is initiated by external stimuli such as growth factors, and progression through the stages of division is governed by two classes of regulatory molecules, cyclins, and cyclin-dependent kinases [4]. Cyclin-dependent kinases are constitutively expressed in cells whereas cyclins are synthesised at specific stages of the cell cycle [5, 6]. The duration of time spent in different stages of the cell cycle varies, and cell populations divide asynchronously [7]. This asynchronous division results in transcriptional variability between cells. For this reason many studies often go to great effort to synchronize cell cultures, or arrest cells in a specific phase [5, 8].

Transcriptional variability is also particularly significant in cells undergoing differentiation. Through differentiation, stem cells or progenitor cells asymmetrically divide in order to generate many different cell types. For example, a single embryonic stem cell develops into a multicellular organism including muscle cells, brain cells, and skin cells, all of which are characterized by very different transcriptional programs. Asymmetric division results in cells that express different genes, giving them different behaviour and diverging fates [9]. In many cases, the phenotype of stem cells and cells undergoing differentiation is not well defined. For example, current state-of-the-art enrichment strategies result in a population of hematopoietic stem cells that is approximately 50% pure as determined by a functional assay [10]. Therefore, bulk analysis of hematopoietic stem cells obscures the relevant sub population.

Transcription differences between cells can also arise due to the onset of disease. Diseases such as cancer often have their origin in a single cell [11]. Environmental exposures, or the accumulation of genetic mutations through multiple cell lineages
can lead to pronounced heterogeneity in the tumor cells which is manifest as aberrant gene expression [12–14].

1.3 Types of Transcripts Defining Cellular State

The two classes of RNA transcripts that determine cell state are messenger RNA (mRNA), which code for proteins, and non-coding RNA such as microRNA (miRNA) which act as regulators of gene expression.

mRNA is transcribed from DNA, and genetic information is encoded in the sequence of nucleotides. This nucleic acid is translated into protein according to codons, consisting of three bases each, which encode for specific amino acids. mRNA consists of a 5′ cap, a coding region containing the codons for translation, 5′ and 3′ untranslated regions, and a 3′ poly-adenine tail. A typical mammalian cell contains thousands of different types of proteins in varying abundance. Proteins involved in metabolic functions, and structural integrity of the cell are generally found in high abundance, and are often referred to as housekeeping genes. Although less abundant, mRNA transcripts also produce proteins involved in intra- and extracellular signaling, as well as transcription factors. Transcription factors are proteins involved in the process of transcribing DNA into RNA, and play a significant role in regulating gene expression. Transcription factors have DNA-binding domains that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. Some transcription factors bind to a DNA promoter sequence near the transcription start site and help form the transcription initiation complex. Other transcription factors bind to regulatory sequences, such as enhancer sequences, and can either stimulate or repress transcription of the related gene.

Non-coding RNAs such as transfer RNA (tRNA) and ribosomal RNA (rRNA) are known to be essential in translating mRNA into protein. However, several varieties of short, non-coding RNAs such as small nucleolar RNA (snoRNA), and small interfering RNA (siRNA) are increasingly being shown to play important roles in regulating gene expression. In particular, microRNAs (miRNA) have been found to be master regulators of gene expression and are drivers in development and cancer [15]. Discovered in 1993, miRNA are a species of small (approximately 22 nucleotides) non-protein coding RNA. Primary miRNA transcripts are transcribed as stemloop structures that are then processed by a protein complex known as the Microprocessor complex (con-
sisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha) into shorter structures [16]. Further processing is performed in the cytoplasm by the endonuclease Dicer, which cleaves the stemloop to form the mature miRNA [16]. Interaction with Dicer initiates the formation of the RNA-induced silencing complex (RISC), responsible for the gene silencing observed due to miRNA expression and RNA interference [16]. This RISC-integrated miRNA strand regulates gene expression by binding to complementary mRNA molecules and inhibiting translation or inducing degradation (by argonaute proteins of the RISC complex). Hundreds of miRNA species are known in humans, and their short sequence length of the critical 5’ seed region makes them complementary to hundreds of mRNA transcripts that can potentially be targeted for regulation [17]. Recent research has revealed tissue-specific distributions of miRNAs appearing at different stages of mammalian development. In particular, Chen and colleagues demonstrated that overexpression of a select few miRNAs (e.g. miR-181a) can influence hematopoiesis [18], and Calin et al. provided evidence for miRNA involvement in cancer by determining that miR-15a and miR-16a are down regulated in over 68% of chronic lymphocytic leukemia patients [19]. These findings suggest the potential application of using miRNA expression profiles to identify those miRNAs involved in human cancer development. Importantly for the current work, single cell measurements of miRNA in highly purified cell populations have been found to exhibit low cell-cell variability, suggesting that miRNA may be a very useful biomarker of cellular state [20].

### 1.4 Techniques for Single Cell Measurements of Transcription

Early changes in cell state are first revealed in the transcriptome, where quantitative measurements with single molecule sensitivity are possible by both imaging and PCR techniques. This section reviews the current state-of-the-art for single cell measurements of transcription.

#### 1.4.1 mRNA-FISH

Fluorescent in situ hybridization (FISH) is a technique for detecting specific DNA sequences in fixed cells. FISH uses fluorescent microscopy to image fluorescently labeled
probes that bind to DNA with similar sequences. In 1998, Femino et al. modified FISH and digital imaging techniques in order to detect single RNA molecules [21]. Specifically, multiple oligodeoxynucleotide probes were synthesized with five fluorochromes per molecule. The probes, each about 50 nucleotides long, are designed to hybridize to adjacent locations on the mRNA target such that their collective fluorescence becomes visible as a diffraction-limited spot. Single molecules are measured by processing images acquired from a series of focal planes through a hybridized cell. Combinations of these probes labeled with spectrally distinct colours have been used to measure up to 11 genes simultaneously [22]. Raj et al. improved upon this technique by probing each mRNA species with 48 or more short, singly labeled oligonucleotide probes [23], which improved the ability to resolve single transcripts. This technique has been applied in two studies looking at the effect of variable gene expression on cell fate [24] and response [25]. By counting transcripts of the genes in a network in individual embryos (up to 200-cell stage), Raj et al. showed that the expression of an otherwise redundant gene becomes highly variable in mutants and that this variation is subjected to a threshold, producing an ON/OFF expression pattern of the master regulatory gene of intestinal differentiation [25]. Beyond quantifying mRNA in single cells, mRNA-FISH reveals the location of the transcript inside fixed cells [23]. Also, the spatial organization of gene expression among fixed cells can be assessed.

Although mRNA-FISH has been successfully applied [24, 25], the system has not been widely adopted. One reason for this is the difficulty in synthesizing heavily labeled oligonucleotides [23]. Additionally, mRNA-FISH requires a long protocol involving fixing cells, hybridizing probes, washing unbound probes, and taking stacks of images using fluorescent microscopy. This procedure requires highly specialized and expensive equipment and reagents. Processing the stack of focal plane images requires exhaustive deconvolution and is computationally intensive [23]. Furthermore, it is challenging to unambiguously identify all the fluorescent spots as mRNA molecules as it is impossible to determine whether the detection of an individual probe arises from legitimate binding to the target mRNA or from nonspecific binding [23]. The use of multiple probes bound to a single transcript also presents challenges in distinguishing between closely related sequences. Small RNA species, such as miRNAs, are too short to accommodate multiple probes, making them refractory to analysis by FISH. Throughput of mRNA-FISH is limited by cost, labour intensive protocols,
1.4. Techniques for Single Cell Measurements of Transcription

and imaging. However, Raj et al. measure hundreds (exact number not specified) of individual cells inside fixed embryos [25]. Another limitation is the low multiplexing. Although Levsky et al. propose the use of multiple probes of different colours in order to create spectral barcodes that can be used for simultaneously measuring many different genes, the largest multiplexing reported involved 11 different genes [22].

1.4.2 RNA-Seq

The deep coverage provided by next-generation sequencing has recently permitted a direct approach to single cell gene expression measurements by sequencing RNA, known as RNA-Seq or whole transcriptome shotgun sequencing (WTSS) [26]. In RNA-Seq, mRNA is captured on poly(T) coated magnetic beads prior to reverse transcription. The cDNA is then fragmented, size selected, and sequenced [27]. The deep coverage allows expression levels to be estimated based on the extent to which a sequence is detected [28]. RNA-Seq has recently been applied to single cells of the inner cell mass from human embryonic stem cell development [29]. This study looked at expression dynamics of 385 genes in 74 single cells [29]. The primary advantage of RNA-Seq is that it permits analysis of the entire transcriptome. The major limitation to this approach is representation bias, which makes RNA-Seq poorly suited to diagnostics or other applications where the abundance of a given molecular species is in question. Further bottlenecks in single cell transcriptome sequencing are cost (although sequencing costs are rapidly dropping), and sample preparation. In the single cell RNA sequencing studies, small numbers of single cells are laboriously isolated by mouth pipetting, limiting high-throughput application. It is also worth noting that single cell RNA sequencing has only been performed by a single industrial group [26, 29].

1.4.3 Rolling Circle Amplification

Rolling circle amplification (RCA) provides a sensitive and accurate method for highly multiplexed DNA analysis that has been widely applied to high-throughput studies of single nucleotide polymorphisms (SNP) [30–32], and recently applied to transcripts [31, 33, 34]. In RCA, a nick in a double stranded circular piece of DNA (e.g. a plasmid) is used to initiate DNA synthesis by polymerase activity. Continued DNA synthesis can produce multiple single-stranded linear copies of the original DNA in
1.4. Techniques for Single Cell Measurements of Transcription

a continuous head-to-tail series called a concatemer. These linear copies can be converted to double-stranded circular molecules.

RCA uses DNA hybridization probes such as molecular inversion probes and padlock probes. Molecular inversion probes consist of a DNA strand with complementary regions at the 5’ and 3’ ends that flank a transcript, such as a SNP. Upon hybridization with its target strand of RNA, the two target complementary regions at the 5’ and 3’ ends of the probe become adjacent to one another while the internal linker region forms a free hanging loop. Polymerase activity is used to fill the gap in the loop. The internal linker region of the probe contains sequences that can be used for subsequent identification of transcriptions using microarrays, sequencing, or PCR methods [30, 35]. Padlock probes are similar to molecular inversion probes, however there is no gap between the complementary regions of the 5’ and 3’ ends. The 5’ and 3’ complementary regions are typically 20 nucleotides in length, and are connected by a linker sequence of 40 bases. The probe becomes circularized upon hybridization. In 1994, Nilsson et al. reported the first use of padlock probes against a variety of DNA targets, and demonstrated that the probes were able to distinguish between closely related targets [36].

The primary advantage of RCA is that it permits in situ amplification [34, 36, 37], revealing information on the expression distribution across fixed tissue. Larsson et al. developed a protocol that allows for in situ detection of individual DNA molecules based on padlock probes [37]. Recently, this method has been extended to measure mRNA by first performing reverse transcription, followed by RCA targeting the cDNA [31]. Larsson et al. detected a somatic point mutation, differentiated between members of a gene family and performed multiplex detection of transcripts in human and mouse cells and tissue [31]. The multiplexing was limited to 3 gene transcripts [31].

The application of RCA to single cell transcript analysis is limited by the low throughput, complex protocol, specialized reagents, and difficulties detecting small RNAs [33]. This is noticeable in the study by Larsson et al., where the reaction steps include fixing the tissue sample, an overnight reverse transcription, degrading the mRNA prior to RCA, and image processing [31]. Furthermore, Larsson et al. report detecting only 30% of available β-actin transcripts (compared to RT-qPCR data), indicating that RCA has a low efficiency [31].
1.4. Techniques for Single Cell Measurements of Transcription

1.4.4 PCR Methods

1.4.4.1 Real-Time Quantitative PCR

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) provides a powerful and sensitive method for quantitative analysis of transcript levels, and has been extensively applied to single cell analysis [38–45]. RT-qPCR is based on the traditional polymerase chain reaction (PCR), which is a method for specifically and exponentially amplifying DNA starting from as little as a single copy [46]. In RT-qPCR, the first strand of DNA is synthesized from a RNA template through a process called reverse transcription (RT). Oligonuclotides (primers) designed to be complementary to the transcript of interest are used to specifically transcribe the RNA into a complementary DNA (cDNA). After first strand synthesis, real-time quantitative PCR (qPCR) is performed similar to conventional PCR, however a fluorescent reporter probe is added to the reaction. During the annealing stage of the PCR, both probe and primers anneal to the DNA target. A variety of molecular probes have been developed for RT-qPCR including intercalating dyes [47], molecular beacons [48], scorpion probes [49], and hydrolysis probes. Fluorescence Resonance Energy Transfer (FRET) probes are perhaps the most commonly used [50]. These probes consist of a dual labeled DNA oligo, having a fluorophore and a quencher at the 5’ and 3’ ends respectively, that is complementary to an internal region of the amplicon. In close proximity, the quenching molecule prevents detection of the fluorescent molecule by absorbing energy from the reporter through a process called Forster resonance energy transfer (FRET) [51, 52]. Following annealing, the polymerization of a new DNA strand is initiated from the primers. Upon reaching the oligonucleotide of the probe, the nuclease activity of the polymerase degrades the probe, physically separating the fluorophore from the quenching moiety, and resulting in an increase in fluorescence. Fluorescence is detected through the use of photodetectors or a charge coupled device (CCD). Monitoring the fluorescent signal of the PCR reaction allows for quantitative measurements of transcript levels [53, 54].

The specificity, sensitivity, dynamic range, and quantitative accuracy make RT-qPCR the most common technique for gene expression analysis [55]. RT-qPCR is sensitive enough to detect transcripts at the level of single cells, and a number of different strategies for single cell RT-qPCR have recently been reported [38–41, 43–45, 56]. Bengtsson et al. used RT-qPCR to reveal lognormal distributions of mRNA
in single cells of pancreatic islets of Langerhans [38]. However, the application of RT-qPCR to large numbers of single cells has been limited in part due to the high cost of probes and reagents. Additionally, laborious techniques such as mouth pipetting, micropipetting, and FACS are used to isolate single cells for RT-qPCR reactions [43, 44]. The latter, although automated, requires careful optimization and calibration which make it difficult or impossible to confirm single cell capture. RT-qPCR is able to target small RNAs, such as miRNAs, through the use of a stem-loop RT primer that yields a longer cDNA strand for annealing qPCR primers and probes [57]. This stem loop primer system has been used to perform highly multiplexed miRNA transcript measurements in single embryonic stem cells [58, 59].

1.4.4.2 Digital PCR

Microfluidic lab-on-chip technology has enabled digital PCR (dPCR), whereby single DNA molecules are quantified by compartmentalizing a sample into thousands of nano- or pico-liter PCR reactions. The sample is diluted such that each reaction chamber has a high probability of containing 1 or 0 molecules. After PCR in the presence of a fluorescent probe, each reaction chamber in the array will be either fluorescent if the PCR reaction was successful, or not fluorescent if the reaction did not occur (i.e. no DNA template present). An end-point image of the array of reaction chambers can be used to read the detection of DNA in a on/off (digital) format. Digital PCR has been applied to quantify transcription factors in a limited number of single cells, following FACS isolation of single cells and RNA processing (lysis, RT reaction) in tubes [60]. The small reaction volumes in dPCR provide a 1000-fold reduction in reagent consumption cost, while providing single molecule sensitivity. However, the bottleneck in single cell dPCR remains laborious single cell isolation and sample preparation.

1.5 Integrated Microfluidic Technology for Single Cell Gene Expression Analysis

Microfluidic systems offer a number of advantages for single cell analysis of gene expression. A challenge in single cell RT-qPCR is the limited starting material [44]. Microfluidics improve reaction sensitivity by reducing the volume of reactions, thereby
1.5. Integrated Microfluidic Technology for Single Cell Gene Expression Analysis

Increasing the concentration of template. Reducing reaction volumes also decreases costly reagent consumption. Microfluidic devices are automatable and highly scalable, permitting high-throughput and cost-effective application. Furthermore, the precise fluid handling capability of microfluidic systems is ideal for delicate manipulation of single cells and the assembly of reactions with low technical variability.

1.5.1 Multilayer Soft Lithography

The microfluidic technology developed in this thesis is based on a fabrication technique called multilayer soft lithography (MSL). In MSL, silicon wafers covered in photoresist are exposed to UV light through a micro-patterned photomask. This mask determines the pattern of features on the wafer after the resist is developed. For example, a typical negative resist such as SU8 consists of a non-photosensitive substrate, a photosensitive cross-linking agent, and a coating solvent. Crosslinks form when the photoresist is exposed to UV light, and the resist polymerises. This exposed photoresist is now insoluble in a developer solution, while unexposed sections of the photoresist are subsequently washed away by the developer. Using different photoresists (and coating spin speeds), wafers can be fabricated with features of varying heights and shapes.

These patterned wafers are used as replica molds for slabs of polydimethylsiloxane (PDMS) that are stacked on top of each other. Replica molding allows low cost production of multiple chips from a single silicon master. Bonding between layers is achieved by complementary off-ratio stochiometric mixing of the potting prepolymer component (A) and hardener component (B) of the room temperature vulcanizing PDMS for each slab. For example, the normal stochiometric ratio of masses A:B is 10:1. Bonding can be achieved between PDMS layers of A:B components, such as 20:1 and 5:1.

A simple microfluidic device can be created from a ‘control’ wafer, and a ‘flow’ wafer[61]. A thick slab of PDMS (with excess hardener) molded to the features of the ‘flow’ wafer can be peeled from the ‘flow’ wafer and bonded to a thin layer of PDMS (with excess potting prepolymer) molded to the ‘control’ wafer. After bonding, this double-slab of PDMS can be peeled from the ‘control’ wafer, punched with holes for fluid inlets/outlets, and bonded to a blank layer of PDMS to close the bottom of the ‘control’ layer channels. Applying pressure (controlled off-chip by solenoids) on the fluid in a control line can deflect the membrane between orthogonally crossing...
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Figure 1.1: Valves in MSL. (A) A schematic profile of valve geometry in MSL devices. (B) Applying pressure to fluid in a ‘control’ channel deflects the membrane with the ‘flow’ channel to effectively valve the ‘flow’ line. These valves can integrated into devices as a peristaltic pump, shown in inverse microscope images with all valves off (C), and one valve on (D).

channels in the adjacent ‘flow’ layer, effectively valving the flow channel (Figure 1.1). Microfluidic devices integrate thousands of these valves (100 $\mu$m x 100 $\mu$m) in order to partition channels, direct fluid flow, and build active structures such as peristaltic pumps and mixers [61, 62].

1.5.2 Review of Microfluidic Technology for Single Cell Analysis

The past decade has seen a surge of efforts to manipulate and analyze individual cells in controllable lab-on-a-chip devices [63]. As microfluidic technology has matured, many of the functionalities required for single cell gene expression analysis, such as cell trapping or nucleic acid detection, have been demonstrated in forms ranging from proof-of-concept to commercial products [64]. Fitting these pieces of the puzzle
1.5. Integrated Microfluidic Technology for Single Cell Gene Expression Analysis

together into a single integrated microfluidic device for high throughput, cost effective single cell gene expression analysis remains the current challenge.

1.5.2.1 Cell Manipulation

Single cell manipulation and isolation is a task well suited to micro-scale devices, and a number of distinct strategies have been demonstrated. In particular, physical trapping, encapsulation in droplets, and dielectrophoresis trapping techniques show great potential for single cell analysis.

Physical trapping of single cells in microfluidic devices has been accomplished through integration of microwells [65], cups [66–70], weirs [71, 72], and active valving [73–75]. Wheeler et al. designed a microfluidic device capable of passively isolating and trapping a single cell from a bulk suspension by positioning a square-cup “dock” with small drain channels at the stagnation point of a T-junction [70]. Similar strategies have explored different cup shapes [69, 72], and densely arraying the traps for large-scale experiments [67]. Skelley et al. developed a device for cell pairing that featured 6,000 physical cell traps made of polydimethylsiloxane (PDMS) [76]. The cell traps were densely arrayed (in an area of 8 mm by 4 mm) within a flow-through channel. Each cell trap consisted of a capture cup, and support pillars on either side of the capture cup to allow flow into and under the trap. The pillar heights were designed to be slightly smaller than the cell diameter in order to trap a cell upon entering the capture cup. The obstruction provided by a trapped cell impedes fluid flow through the cell trap resulting in subsequent cells flowing past to be captured by unoccupied cell traps. Skelley et al. observed that the trap spacing in the array was critical for efficient capture without clogging. With optimal column spacing of 1-1.5 cell diameters (~20 µm), and a row spacing of 20-50 µm, Skelley et al. captured 70 - 90% of cells entering the array [76]. This physical cell-trapping array is a highly parallel and scalable technique.

Microfluidic technology for generating monodisperse droplets of aqueous phase solution inside an inert oil have been applied to the encapsulation single cells. Once encapsulated inside a droplet, the droplet acts as an individual test tube, and reagents (other droplets) can be combined, and reactions can be carried out. Koster et al. developed a microfluidic device to encapsulate individual cells in picoliter aqueous drops in a carrier fluid at rates of up to 250 Hz [77]. In addition to cells remaining viable for up to 6 hours incubating inside 33 pL droplets, the small volumes of the
drops enables the concentrations of secreted molecules such as antibodies to rapidly attain detectable levels. One limitation to this system is variability in the number of cells per drop due to stochastic cell loading. However, Edd et al. solved this issue by designing a high aspect-ratio microchannel that hydrodynamically focuses cells to be evenly spaced as they travel within the channel [78]. Thus, individual cells enter the drop generator with the frequency of drop formation. Encapsulation of cells within picolitre-size monodisperse drops provides new means to perform large-scale quantitative biological studies on a single-cell basis.

Microfluidic devices integrated with active electronic electronics have been used to manipulate cells with electric fields. This approach offers the advantage that the cells are not physically contacted. In dielectrophoretic cell trapping, a non-uniform electric field is generated, and the force applied to the cell depends on the dipole induced within the cell. Voldman et al. used four monolithic pillars within a microfluidic channel as electrodes to create a quadropole dielectrophoresis cell trap [79]. Dielectrophoresis can be selective in only trapping particular cell types, such as selecting white blood cells instead of erythroctyes [79]. In addition, the traps can be switched ‘on’ or ‘off’ to facilitate cell recovery or subsequent manipulation. Alternatively, optical tweezers have also been combined with microfluidic devices for single cell manipulation [80–82].

1.5.2.2 RNA Processing

Techniques such RT-qPCR and sequencing often require RNA manipulations including purification or reverse transcription into cDNA before further analysis. Single cell capture, lysis, and reverse transcription have been implemented in a microfluidic rotary mixer that may be injected with cell sample, reagents, and output RT product [73]. The throughput is limited to one cell, however. Bontoux et al. applied this system to neuronal progenitors, followed by template switching PCR in a tube, and reported the detection of 5000 genes in each cell (corresponding to the expected total number of genes expressed) by microarray analysis [73]. However, due to the low reported correspondence between different cells and the lack of data analysis it is unclear how much of this signal was specific. Interestingly, Bontoux et al. reported that the RT reaction was more efficient in nanoliter volumes inside the microfluidic device compared to microliter volume reactions in tubes [73].

Zhong et al. reported a microfluidic device capable of purifying mRNA from
20 single cells using oligo(dT) beads, followed by recovery for off-chip qPCR [83]. Individual cells were stochastically isolated by partitioning a cell suspension between physical microvalves. A chemical buffer was mixed with the sample fluid to lyse the cell. The cell lysate was pushed through a column of beads functionalized with short sequences of deoxy-thymine nucleotides on the surface. The oligo(dT) strand binds the poly-A tail of mRNA transcripts, thereby capturing the mRNA from the cell while remaining contents are washed away. By performing reverse transcription of the purified mRNA on the microfluidic device, Zhong et al. demonstrated a 4-fold increase in reverse transcription reaction efficiency (measured by cDNA yield) compared to performing the reaction in conventional tubes [83]. Measurement of 3 transcripts in 54 single hESCs revealed a heterogeneous population [83], further underscoring the need for discrete cell analysis. The throughput of this system is limited by stochastic cell loading, and challenges recovering the samples from the device for off-chip analysis.

1.5.2.3 Transcription Analysis

Microfluidic devices employing arrays of thousands of nanolitre micro-reactors have been used by researchers for highly multiplexed, as well as single molecule quantitative analysis of cDNA prepared from single cell samples. Digital PCR is performed in a microfluidic “Digital Array” whereby a 7.5 µl sample is partitioned into 1,200 isolated reaction chambers (“wells”), before PCR [60]. The sample is diluted such that each reaction chamber has high probability of containing a single template molecule, or zero, allowing absolute quantification of single molecules by counting the number of fluorescent reaction wells after PCR amplification. Warren et al. used FACS to sort 116 individual cells using hematopoietic differentiation markers to select cells representing hematopoietic stem cells (HSC), common lymphoid progenitors (CLP), two sub-populations of common myeloid progenitors (CMP), and megakaryocyte-erythroid progenitors (MEP) [60]. Following off-chip reverse transcription, abundance of transcription factor PU.1 was quantified using digital PCR. Warren et al. were able to show differential expression of PU.1 between flk+ and flk- CMPs with single cell (and single molecule) resolution.

Integrated fluidic circuits for digital PCR have been commercialized by Fluidigm, which also offers a Dynamic Array chip for quantitative analysis by highly multiplexed real-time PCR. Following conventional (off-chip) sample preparation (including re-
verse transcription and pre-amplification of cDNA), the Dynamic Array combines 48 samples with 48 assays, to perform 2,304 real-time PCR reactions, each 10 nL in volume. In addition to fluid handling advantages, performing the equivalent multiplexing real-time PCR experiments in conventional microliter volumes quickly becomes cost prohibitive. This multiplexing allows for large-scale gene expression profiling, starting with small samples such as single cells.

The Fluidigm Dynamic Array technology has been leveraged to study cellular development from zygote to blastocyst stage fertilized mouse embryos [84]. Guo et al. investigated expression of 48 genes in a survey of 500 single cells from 8, 16, 32, and 64 -cell stage embryos [84]. By tracking multiple expression markers, Guo et al. revealed at least three distinct developmental expression patterns, and associate these with development of cells forming the trophoderm (TE), the primitive endoderm (PE), and the epiblast (EPI) [84]. Furthermore, Id2 and Sox2 were identified as the earliest markers of outer and inner cells, respectively. These results illustrate the power of single cell gene expression analysis to provide insight into developmental mechanisms, and this technique is applicable to other biological systems.

1.5.2.4 State of the Art: Integrated Systems for Cell Manipulation and RNA Analysis

The above examples demonstrate the single cell handling, nucleic acid processing, and analysis capabilities of microfluidic devices, however complete integration of all sample processing and analysis into a single device remains a pursuit of active research. Notably, Toriello et al. developed an integrated microfluidic device for single cell gene expression capable of capturing a single cell, cell lysis and reverse transcription of contained mRNA, followed by amplification and detection of product of interest [85]. The device features a nanoliter metering pump, and DNA capture pads to catch functionalized single cells. An integrated heating element is used for cell lysis, followed by RT-PCR. The 200 nL PCR chamber is coupled to capillary electrophoresis for size-based measurement of products. Each device is capable of measuring 4 single cells in parallel, and is used to measure variable siRNA knockdown of the GAPDH gene in 8 individual Jurkat cells [85]. These devices demonstrate the feasibility of a microfluidic approach to single cell expression analysis, however further development is needed for microfluidic based methods to become routine in single cell analysis.
1.6 Research Statement

A major bottleneck in single cell gene expression analysis is the lack of tools for high throughput, cost effective measurements of transcripts in single cells. The specific goals of this research are:

1. Develop an integrated microfluidic device to perform all fluid handling for single cell capture, lysis, reverse transcription, and qPCR
2. Enable high-throughput and cost-effective application through automation, parallel architecture, and miniaturization
3. Apply the microfluidic system to a variety of cell types and targets

The ability to screen isolated cells by simultaneously measuring the fraction of cells expressing a specific transcript, and quantifying the abundance of expression, provides a new modality for the study of heterogeneous systems such as developing tissue undergoing differentiation and disease progression.
References


Chapter 1. References


Chapter 1. References


Chapter 1. References


Chapter 2

High-Throughput Microfluidic RT-qPCR of Single Cells

We present a microfluidic device for precise gene expression measurements of hundreds of single cells. Automated on-chip processing in nanoliter volumes provides improved precision, single molecule sensitivity, and single nucleotide specificity. We apply this technology to single cell studies of i) miRNA expression in K562 cells, ii) co-regulation of a miRNA and one of its target transcripts during differentiation of embryonic stem cells, and iii) SNV detection in primary tumor cells.

2.1 Introduction

Single cells represent the fundamental unit of biology. Despite this the vast majority of molecular biological knowledge has emerged as a consequence of studying cell populations and not individual cells. Inevitably, fundamental and applied questions, such as those relating to transcriptional control of differentiation, intrinsic noise in gene expression, and the origins of disease, can only be addressed by analyzing transcript expression in large numbers of single cells. Here we couple the scalability and precision of microfluidics with the sensitivity of RT-qPCR to establish a robust, low-cost, and simple method for performing precise gene expression measurements of hundreds of single cells per run. Integrated cell processing in nanoliter volumes provides improved measurement precision, single molecule sensitivity, and single nucleotide specificity, opening the door to routine and automated analysis of transcriptional heterogeneity in research and clinical settings.

Current methods for measuring transcript levels in single cells include RT-qPCR [1], single molecule counting using digital PCR [2] or hybridization probes [3, 4],

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1 A version of this chapter has been submitted for publication. Adam White, Michael VanInsberghe, Oleh Petriv, Mani Hamidi, Darek Sikorski, Marco A. Marra, James M. Piret, Sam Aparicio, and Carl L. Hansen, *High-Throughput Microfluidic RT-qPCR of Single Cells*, 2010.
and next generation sequencing [5]. Of these, single cell RT-qPCR provides combined advantages of sensitivity, specificity, and dynamic range but is limited by low throughput, high reagent cost, and difficulties in accurately measuring low abundance transcripts. Microfluidic systems offer a potential solution to these problems by providing economy of scale, automation and parallelization, and increased sensitivity in small volume reactions. In recognition of this potential, the basic functionalities for single cell gene expression analysis on chip have been previously developed, including cell manipulation and trapping [6], RNA purification and cDNA synthesis [7], and qPCR [8]. However, despite considerable effort and impressive advances in microfluidic integration [9], the realization of scalable and precise single cell transcription measurements on chip has to date been elusive.

2.2 Results

2.2.1 A Microfluidic Device for Single Cell RT-qPCR

Here we describe a simple device that performs 300 parallel RT-qPCR assays and executes all steps of single-cell capture, lysis, reverse transcription, and qPCR (Figure 2.1). The device features 6 sample input channels, each divided into 50 reaction chambers (60.6 nL each), resulting in 300 RT-qPCR reactions using a total of approximately 20 µL of reagents. Each unit consists of a reagent injection line, a 0.6 nL cell capture chamber with integrated cell traps, a 10 nL RT chamber, and a 50 nL PCR chamber. Each trap includes upstream deflectors to direct cells into the capture region. A single cell suspension is injected into the device. Cell traps isolate single cells from the fluid stream and permit washing of cells to remove extracellular RNA. Actuation of pneumatic valves results in single cell isolation prior to heat lysis. Injection of reagent (green) for reverse transcription (RT) reaction (10 nL). Reagent injection line is flushed with subsequent reagent (blue) for PCR, prior to injection. Reagent for qPCR (blue) is combined with RT product in 50 nL qPCR chamber. Scale bar for D-I: 400 µm.
2.2. Results
2.2. Results

Figure 2.2: Single cell loading and amplification. (A) The locations of cells in each chamber along two lanes of a device, as determined by brightfield microscopy, are represented as white circles and overlaid on a heat map of CT values obtained from RT-qPCR measurements of miR-16. Region identified in (B) is indicated with white bars. (B) Fluorescent image of lanes depicted in (A) taken after 32 cycles of PCR. (C) Expanded view of section of array in (B) showing correspondence of amplification signal with cell localization. Cells are visible as punctuate fluorescent spots adjacent to each chamber.

2.1A). To resolve technical pitfalls and achieve robust and precise single cell measurements we designed our device to 1) allow for efficient loading of single cells without mechanical damage, 2) avoid reaction inhibition by cell lysates in nL volumes, and 3) minimize dehydration of reagents during thermocycling. A key element of our technology is the integration of hydrodynamic cell traps within arrays of multistep nanoliter volume reactors. (Figure 2.1C). Cell traps were designed with upstream deflectors to achieve highly efficient chamber loading, resulting in the successful isolation of single cells in 2241 of 2650 chambers (85%) over 12 separate experiments (Figure 2.2). Traps also assist in reducing background signal by enabling the washing
2.2. Results

Figure 2.3: Microfluidic qPCR real-time amplification curves. (A) Fluorescence image of entire device showing 300 reactions in 6 lanes. Image is taken after 40 cycles of PCR from dilution series of purified total RNA. From left to right the samples are NTC, 10 fg/chamber, 78 fg/chamber, 625 fg/chamber, 5 pg/chamber, and 40 pg/chamber. Single molecule amplification at limiting dilution results in a digital amplification pattern for 10 fg and 78 fg lanes. (B) 300 real time amplification curves generated from processing sequences of images similar to (A).

of cells immediately prior to analysis. To reduce complexity and obviate the need for RNA purification, we optimized our device to be compatible with commercially available assays using one-pot RT-qPCR chemistries. Each 0.6 nL cell capture chamber is connected sequentially to two larger chambers having volumes of 10 nL and 50 nL respectively (Figure 2.1B), to allow the implementation of heat lysis followed by two-step RT-PCR (Figure 2.1D-I), or chemical lysis followed by one-step RT-qPCR. Temperature control and fluorescence detection during amplification is performed using a CCD detector mounted above a flat-bed thermocycler plate (Figure 2.3).

We first evaluated the sensitivity and precision of our device by performing measurements of GAPDH expression over an 8-fold dilution series of total RNA, ranging from 40 pg (~2 cell equivalents) to 10 fg (~1/2000 cell equivalents), purified from K562 cells (Figure 2.4). For the three highest template amounts (40 pg, 5 pg, 625 fg), mean cycle threshold (CT) values were found to differ by 2.995 and had standard deviations of less than 0.5, indicating uniform and approximately 100% efficient amplification
2.2. Results

Figure 2.4: On-chip RT-qPCR for GAPDH is quantitative over a serial dilution of total RNA purified from K562 cells. Error bars represent standard deviation (N=50).

across the array. Template amounts below 625 pg resulted in a digital pattern of single molecule amplification (40/50 for 80 fg, 11/50 for 10 fg) corresponding to an average copy number of 440 copies per single cell equivalent (20 pg) (Figure 2.4). This measurement, which does not account for RT efficiency, is comparable to previous reports [7] and is also consistent with estimates based on normalizing the dilution series to CT values obtained for single molecules (630 ± 220 copies).

To evaluate the efficiency and reliability of on-chip cell processing, we next compared these results to measurements performed directly from single K562 cells (Figure 2.5). Using a chemical lysis and one-step RT-qPCR protocol (Cells Direct™, Invitrogen) we observed a log-normal distribution of GAPDH with mean CT values of 23.9 (s.d. = 1.3) and an average copy number of 103 (s.d. = 63) copies per cell, consistent with previous estimates on a related cell type [2]. Assuming 20 pg of RNA per cell, we conclude that the efficiency of one-pot mRNA extraction and RT is approximately 25%. This was found to be consistent with reactions performed in 20 µL volumes. As expected, RT-qPCR measurements from chambers loaded with more than one cell show less variability and lower CT values (Figure 2.6). Taken together these results establish the precise measurement of mRNA abundance with single molecule
2.2. Results

Figure 2.5: Single-cell measurements of GAPDH expression in K562 cells (N=174).

sensitivity and the dynamic range needed for single cell analysis.

2.2.2 miRNA Expression Measurements in Single Cells

We then applied our technology to the analysis of single cell miRNA expression. miRNAs are thought to provide a unique signature of cellular state and are central players in orchestrating development and oncogenesis, making them a promising class of biomarker for single cell analysis [10–12]. Importantly, the short length of miRNAs (~22 nucleotides) makes them difficult to detect by hybridization approaches so that RT-qPCR is the preferred quantification strategy. To demonstrate the robustness and throughput of our technology, we performed a total of 2067 single cell measurements to examine single-cell variability in the expression of 11 miRNAs spanning a wide range of abundance. K562 cells, a line derived from chronic myelogenous leukemia, were chosen as a heterogeneous population for this study since they exhibit
2.2. Results

Figure 2.6: Measurement of miR-16 in hESC cell aggregates demonstrates that the number of cells is reflected in corresponding cycle threshold (CT) values.

mixed characteristics of erythrocytes, granulocytes, and monocytes [13]. The precision of microfluidic RT-qPCR revealed that miR-16 expression is also log-normally distributed and is very tightly regulated in this population (mean CT = 22.0, s.d. = 0.5), with variability approximately two-fold lower than measurements of GAPDH expression. By comparison, matched experiments performed in 20 µL volumes showed artificially higher variability arising from increased technical error (mean CT = 29.5, s.d. = 0.88) (Figure 2.7A). In contrast to miR-16, miR-223 expression was found to be abundant and highly variable in K562 cells (Figure 2.7B), consistent with the role of this miRNA in myeloid differentiation. Similarly, single cell measurements of the 9 other miRNAs (11 total) (Figure 2.8) revealed distinct patterns of expression with miR-16, miR-92, miR-27a, and miR-17-5p exhibiting unimodal and tightly regulated distributions, while miR-223, miR-196a, and miR-181a show multi-modal distributions and high heterogeneity. To further illustrate the utility of single cell measurements in assessing differences in expression and heterogeneity between two cell types, we compared the expression levels of miR-16 and miR-223 to those in
human embryonic stem cells (hESC). miR-16 was found to be expressed in hESC at similar levels to K562 but with approximately 2-fold larger variability (mean CT = 22.3, s.d. = 0.86) (Figure 2.7A), while miR-223 was strongly down-regulation in hESC and displayed at least three distinct subpopulations of cells.

2.2.3 Co-regulation of Pluripotency Marker by miRNA in Human Embryonic Stem Cell Differentiation

We speculated that the measurement of multiple transcripts in single cells would enable quantitative measurements of gene co-regulation that would otherwise be masked by cellular heterogeneity. To demonstrate this capability we designed an optically multiplexed assay to study the co-regulation of miR-145 and Oct4, a known target of miR-145 [14], during the differentiation of hESCs (Figure 2.9). A total of 547 single cell measurements were performed at 0, 4, 6, and 8 days of differentiation. Cell distributions at each time point were used to map out the evolution of these transcripts and show that average miR-145 levels increase approximately 300 fold over the 8 days. Increases in miR-145 were accompanied by progressive down-regulation of Oct4, ultimately reaching an average of 30-fold suppression after 8 days (independently verified by mRNA-FISH) (Figure 2.10). Interestingly, single cell analysis at day 6 shows a bimodal distribution in both Oct4 and miR-145, revealing a bi-stable switch-like transition of cellular state. This behavior is only visible by single cell analysis.

2.2.4 SNV Detection in a Primary Breast Cancer Sample

Finally, to establish the specificity of our method we used multiplexed measurements of mRNA single nucleotide variants (SNV) to assess the genomic heterogeneity within a primary tumor sample. A total of 117 single cells isolated from a plural effusion of a metastatic breast cancer were assayed for the expression of a SNV mutant of the transcription factor SP1 that had previously been identified by deep sequencing [15] (Figure 2.11). Of the 117 cells analyzed, we detected both mutant and wildtype alleles in 23/117 cells (20%), with the remaining 94/117 (80%) having a homozygous normal genotype. We did not detect the SP1 mutation in 37 control K562 cells and failed to detect the wild-type transcript in only 2 cells. Given that the frequency of tumor cells within the original sample was approximately 89% [15], we conclude that
the metastasis of this tumor is derived from multiple cancer cell lineages.

2.3 Conclusions

In summary, we have described a simple microfluidic device that performs 300 high-precision single cell RT-qPCR measurements per run. We have demonstrated this method in the study of miRNA expression, transcript regulation, and genomic heterogeneity in both adherent and suspension cell lines as well as from clinical samples. We contend that the simplicity of the device operation will allow for the first robust and automated implementation of single cell RT-qPCR, leading to wide adoption and opening the prospect of diagnostic tests based on single cell analysis. Scaling the throughput of this technology to several thousand measurements per run is straightforward and in the near term we anticipate that more complex fluid routing [16] will allow for multiplexed measurements of tens of targets across hundreds of cells. Ultimately, the coupling of integrated on-chip cell processing with advances in high-throughput sequencing will open the door to routine and scalable single cell genomics.

2.4 Author Contributions

AW and MV designed and fabricated microfluidic devices. AW, MV, OP, and MH performed on-chip experiments and analyzed data. MV developed image analysis code. OP and MH developed Oct4 assays and performed off-chip experiments. DS performed hESC differentiation cultures and mRNA FISH measurements. CH, SA, MM, and JP designed research. AW and CH wrote the manuscript.

2.5 Acknowledgements

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2.6 Methods

2.6.1 Device Fabrication and Operation

The microfluidic devices were fabricated by multilayer soft lithography [17, 18]. Planar silicon molds were defined by photolithography, using photomasks designed with CAD software (AutoCAD, Autodesk Inc.), and printed at a resolution of 20,000 dots per inch on transparency films (CAD/Art services). The control mold was fabricated using SU8-2025 photoresist (Microchem, USA) to deposit valve features 24 µm in height. The flow mold was fabricated with three lithographic steps. First, the channels for reagent injection, and connections between chambers were fabricated using 13 µm high SPR220-7 photoresist (Shipley, USA). The SPR channels were rounded to facilitate valve closure by incubation at 115°C for 15 minutes. A hard bake at 190°C for 2 hours was used to prevent SPR photoresist erosion during addition of subsequent layers. Second, the cell trap features were defined in 14 µm SU8-2010 photoresist (Microchem, USA). Finally, the large chambers and fluidic bus lines were constructed using 150 µm high SU8-100 photoresist. All photoresist processing was performed according to manufacturer specifications.

Microfluidic devices were cast from these molds in polydimethylsiloxane (PDMS, RTV615, General Electric, USA). Each device employs push-up valve geometry and consists of a three layer elastomeric structure. The molds were first exposed to chlorotrimethylsilane (TMCS, Aldrich) vapor for 2 min to promote elastomer release after the baking steps. For the flow layer of the device, a mixture of PDMS (5 parts RTV615A : 1 part RTV615B) was poured onto the flow mold. After degassing, the flow molds were baked for 60 min at 80°C. For the control layer of the device, a mixture of PDMS (20 parts RTV615A : 1 part RTV615B) was spun on the control mold at 1800 rpm and baked for 45 min at 80°C. The flow layer was separated from the flow mold and flow channel access holes were then punched. Next, the flow and control layers were aligned and baked for 60 min at 80°C. The bonded, two layer structure was peeled from the control mold, control channel access holes were punched, and the device was mounted to another thin PDMS layer made by spinning 20 : 1 PDMS mixture (2000 rpm) on a blank wafer. After baking for 3 hours at 80°C, the three layer structure was bonded to a clean glass slide and baked overnight at 80°C.

The device operation requires active control of 9 pneumatic valves and may be operated using a simple manifold of manual valves. For the current study a semi-
automated implementation was used in which microfluidic valves were controlled by solenoid actuators (Fluidigm Corp., San Francisco) controlled through a digital input output card (NI-DAQ, DIO-32H, National Instruments) operated using LabView drivers (National Instruments).

2.6.2 Image Analysis

Fluorescence images of the entire device taken in at least two different colors (one passive reference dye and one or more reporter dyes) after each PCR cycle were analyzed using custom scripts written in MATLAB (MathWorks) to generate real-time amplification curves. Using the initial image, the reaction chambers were segmented from the rest of the image using the passive reference dye. First, the image was manually rotated so that all of the reaction chambers were square with the edges of the image. Next, the average image intensities across each row and column were calculated and a threshold was manually set to differentiate bright areas from background. Regions containing both bright rows and bright columns corresponded to reaction chambers.

All subsequent images were automatically aligned to this initial image using phase correlation of Harris corners. For each image, the intensities of the reporter and passive dyes were recorded for each reaction chamber. Real time amplification curves were generated by normalizing the intensity of each reporter dye to that of the passive dye. The baselines were corrected by subtracting the average value of the pre-exponential region of each curve from the recorded intensities. The threshold for determining CT values was defined to be 20 times the standard deviation of the corrected baseline.

2.6.3 Cell Culture

K562 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

CA1S hESCs [19, 20] were propagated in mTeSR [21] basal medium with supplements (STEMCELL Technologies, Inc., Vancouver, BC, Canada), additionally supplemented with antibiotic-antimycotic (100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/mL amphotericin B) (Invitrogen, Carlsbad, CA, USA). Upon passaging, hESCs were washed with phosphate-buffered saline (PBS) prior to incubating with TrypLE Express (Invitrogen, Carlsbad, CA, USA) at 37°C for 10 minutes to detach single hESCs from 4-8 day-old cultures depending on confluency. TrypLE Express
was neutralized with mTeSR1 supplemented with antibiotic-antimycotic and suspen-
sions were then transferred into new tissue culture dishes containing a precoated layer
of 1:30 diluted Matrigel (Becton Dickinson, San Jose, CA, USA) and mTeSR1 supple-
mented with antibiotic-antimycotic. For differentiation, mTeSR1 was replaced with
Dulbeccos minimal essential medium with 10% fetal bovine serum (FBS) 1 day after
plating cells.

When harvesting hESCs for qRT-PCR, cells were instead incubated with TrypLE
Express (Invitrogen, Carlsbad, CA, USA) at 37°C for 20 minutes in order to produce
a more uniform single cell suspension from 4-8 day-old cultures.

Cryo-vials of primary cells isolated from a lobular breast cancer metastasis were
provided by Dr. Samuel Aparicio (BC Cancer Agency, UBC). To increase viabil-
ity, cells were transferred to fresh culture medium and incubated for 2 days before
analyzing in the microfluidic device.

### 2.6.4 Single-Cell RT-qPCR Experiments

The cell loading lines of the microfluidic device were primed with PBS containing
1 mg/mL bovine serum albumin (BSA) in order to prevent cells from sticking to
channel walls.

Cells were lysed in the presence of RNAse inhibitor by heat (7 min at 85°C). Re-
verse transcription was performed using the High Capacity cDNA Reverse Transcrip-
tion kit (Applied Biosystems, USA), and a pulsed temperature protocol (60 cycles of
30 sec at 42°C and 1 sec at 50°C). Real-time qPCR reactions used the TaqMan PCR
Universal Master Mix and TaqMan microRNA assays (Applied Biosystems, USA)
[22]. A surfactant (0.1% Tween 20) was added to RT and qPCR reagents.

Oct4 (POU5F1) primers were as found in RTPrimerDB (ordered from Biosearch
Technologies Inc). Forward primer: ACC CAC ACT GCA GCA GAT CA. Reverse
primer: CAC ACT CGG ACC ACA TCC TTC T. TaqMan probe: Quasar670-CCA
CAT CGC CCA GCA GCT TGG-BHQ-2. RT primer: TTG TGC ATA GTC GCT
GCT TGA T.

The BHQ-Plus probes used for SNV detection (Biosearch Technologies Inc) have
enhanced duplex stabilization allowing for shorter sequence lengths and increased
specificity for SNP/SNV detection. The SNV location for the SP1 locus is from
Table 1 in Shah et al. [15]. Two hundred bp flanking this location on the hg18
sequence were used for assay design. The resulting primer and probe sequences are
as follows (the SNV is underlined):

1. SP1 Mutant Probe: FAM-AGGCCAGCAAAAAAACAAGG-BHQ-1 5’ Modification: FAM, 3’ Modification: BHQ-1 Plus. Tm = 62.7

2. SP1 WT probe: Cal Fluor-CAGGCCAGCAAAAAAGAA-BHQ-1 5’ Modification: CAL Fluor Orange 560, 3’ Modification: BHQ-1 plus. Tm = 62.1

3. SP1 Forward Primer: CCAGACATCTGGAGGCTATTG Tm = 65.8

4. SP1 Reverse Primer: TGAACTAGCTGGAGGCTTGATA Tm = 66.0

Measurements of mRNA transcripts, such as the SP1 SNV, were performed using the Cells Direct kit (Invitrogen, USA). Although mRNA can be measured using the same chemistry and heat lysis protocol used for miRNA assays (e.g. the Oct4 measurements), the Cells Direct kit provides a faster work-flow for mRNA analysis and is compatible with the device architecture. Using the Cells Direct kit, single cells were lysed by injecting a chemical lysis buffer and incubating at 70°C for 10 minutes. The one-step RT-qPCR mix is then combined with the cell lysate into the final 50 nL reaction chamber. The GAPDH assay was obtained from Applied Biosystems. Purified RNA was extracted from K562 cells using RNA MiniPrep (Qiagen, USA).
2.6. Methods

Figure 2.7: Single-cell transcription measurements. (A) Single cell measurements of miR-16 expression in K562 cells and hESCs show tight regulation. Measurements of K562 cells isolated using a microcapillary and assayed in 20 µL volumes are shown for comparison of technical variability. (B) Comparison of miR-223 expression between K562 cells and hESCs. Right-most bar indicates cells for which miR-223 was not detected (ND).
Figure 2.8: 2072 single cell measurements of the expression of 11 miRNA in K562 cells. Reflected histograms represent the expression distributions for each miRNA.
2.6. Methods

Figure 2.9: Multiplexed analysis of the co-expression of Oct4 and miR145 in differentiating hESC. Each point represents a single cell. Histograms showing the distribution of each transcript are projected on the axes.
Figure 2.10: mRNA-FISH of Oct4 (red) counterstained with DAPI (Blue) (A) Representative image of mRNA-FISH of Oct4 in differentiated CA1S cells. Estimate of average copy number of Oct4 mRNA as determined by manual inspection of image stacks is 53 (s.d. = 62, N=3). (B) Representative image of undifferentiated CA1S cells. Estimate of average copy number as determined by manual inspection of image stacks is 860 (s.d. = 200, N=3). Scale bars = 10 µm.
2.6. Methods

Figure 2.11: Co-expression measurements of SP1 wild-type and SNV mutant transcripts in primary cells isolated from a lobular breast cancer sample. Mutant SP1 is detected in 23 of 117 primary cells, and undetected in K562 cells (N=37). Wild-type SP1 is detected in all primary cells and in 35 of 37 K562 cells.
References


Chapter 3

Conclusions and Future Recommendations

The goal of this thesis is the development of a microfluidic system for high-throughput single cell gene expression analysis. This goal is accomplished by integrating cell capture, lysis, and RT-qPCR into a microfluidic device that performs 300 high-precision single cell RT-qPCR measurements per run. A scalable architecture of nanoliter reactors, resulting in a 1000-fold reduction in reagent consumption, enables high throughput application. This technology is applied to measuring miRNA expression in a suspension cell line, the co-regulation of mRNA expression by a miRNA in adherent cells, and detection of SNV mutations in a primary sample. The results presented in this thesis encompass over 3000 single cell gene expression measurements. In addition to improving the throughput of existing microfluidic systems by a factor of 100x [1], this is the first and only demonstration of quantitative single cell gene expression measurements on an integrated microfluidic system.

The particular strengths of this technology are the economy of scale, throughput, sensitivity, dynamic range, and ease of use. The microfluidic device reduces technical noise compared to conventional RT-qPCR performed using micropipettes and tubes. Removing background noise is achieved through single cell trapping, followed by washing the individual cell in the device. In addition to reducing sample preparation, this feature eliminates any extracellular RNA that may obscure cell measurements. Extracellular RNA is inevitably present in cell samples due to cell damage and death. Washing single cells prior to analysis is critical when examining miRNAs, which have been shown to be actively secreted by blood cells, and present in high abundance [2, 3]. Circulating miRNAs have been suggested as potential blood-based markers for cancer detection [2], and microfluidic architecture could be added to the presented microfluidic system to capture secreted miRNAs for measurement.

Quantitative analysis by RT-qPCR benefits from the single molecule sensitivity of the microfluidic system. Transcript measurements by RT-qPCR yield cycle threshold
(CT) values which can be used for comparing expression levels (between different samples, or different targets), but must be calibrated to templates of known concentration in order to extrapolate molecular abundance [4]. Detecting single molecules allows an alternative calibration, where the CT value of a single molecule can be used as the base for calculating the correlating transcript number in reactions.

There is potential to integrate the cell handling and RNA processing of the microfluidic device presented in this thesis with arrays for digital PCR (similar to Warren et al. [5]). This can be accomplished by replacing the 50 nL qPCR chambers in current design with arrays of smaller chambers for partitioning the PCR reaction into digital reactions. Integrating cell processing and digital PCR into a single device would allow transcript abundances to be directly counted for large numbers of single cells. This enables precise quantification of low copy transcripts, and would be useful for studying the frequency of RNA editing events in a population [6], allelic imbalance [7], and other sources of variable transcription that can be significant. One system of interest would be the relative abundance of miRNAs originating from complementary 5’ and 3’ ends of the same pre-miRNA hairpin structure (often denoted miRNA and miRNA*). The function of the miRNA* form is not well understood, with studies showing that it is often selected for degradation, but can also be incorporated into the RISC complex for gene silencing [8].

The cell traps employed in this work have a number of limitations. Although successfully used to isolate suspension, adherent, and primary cells, the use of physical cell traps is influenced by the size of the cells. Cells under 10 µm in diameter, such as hematopoietic stem cells (ND13) and mouse B-cell progenitors (nBaF3), were found to often remain in the fluid stream and bypass the traps, resulting in a low efficiency cell loading. Conversely, overly large cells might result in clogging the microfluidic channels. Robust cell traps able to isolate a variety of cell sizes are important in order to eliminate bias in analyzing a population of single cells. Furthermore, although loading appropriate sized cells resulted in 85% of traps occupied with a single cell, a number of cells make it through the array without being trapped. This is potentially a problem in cases where an extremely limited number of cells are available (such as highly purified samples of rare stem cells). To address this limitation, cell trap and flow-focuser geometries can be optimized for robust handling of more cell sizes, or alternatively for a particular cell sample. Parameters include focuser width, focuser to trap spacing, cup diameter, and the addition of slits in the trap to allow fluid flow.
through unoccupied traps. Alternatively, with additional fluid routing, un-trapped cells can be re-circulated in the device until successfully trapped. This is the subject of ongoing optimization, and the realization of very efficient traps has been achieved [9].

The number of transcripts analyzed on-chip is currently limited by optical multiplexing of the RT-qPCR reaction. The microfluidic system is thus best suited to studying select transcripts of interest, in large numbers of cells. This limitation can be overcome by spatially multiplexing the reaction, through the integration of more complex fluid routing enabled by laser ablation [10]. Additionally, fluidic architecture for recovery of PCR products could be used for subsequent analysis of single cell samples, e.g. by sequencing.

Even without multiplexing, the simplex measurements allow identification of miRNA or mRNA biomarkers that could be used to stratify a cell population. For example, in the measurements of miRNA expression in K562 cells, miR-92, miR-16, and miR-17-5p were all found to be highly abundant in a narrow range of abundance, while miR-223 expression was found to be highly heterogeneous. miR-223 is involved in hematopoiesis, and its variable expression is consistent with K562 cells being able to give rise to erythrocytes, granulocytes, and monocytes [11, 12]. A recent study by Petriv et al. found miRNA expression to be very tightly regulated within highly purified populations of hematopoietic tissue [13]. The microfluidic device presented here could be used to follow up this study by identifying miRNAs that are variably expressed. This allows for assessing the purity of the population, and compartment heterogeneity. Potentially, differentially expressed miRNAs could be used to identify target markers for better purification.

The results from the single cell transcript analysis of hESC cells undergoing differentiation (presented in Chapter 2), suggest further application of this technology to study temporal dynamics of gene expression. The device is well suited to studying co-regulation of select gene transcripts, providing single cell resolution and eliminating the need for synchronizing cell cultures. For instance, measurements of transcript expression in response to signaling stimuli could be measured simultaneously with cyclins to establish the effect of cell cycle on response variability. Future studies could look at co-regulation of additional miRNAs and mRNA targets in embryonic stem cell development. Similarly, measuring the co-regulation between miRNAs could help elucidate their function. For example, the let-7 family of miRNAs and the cell cycle
miRNAs have been shown to act through common pathways to alternatively stabilize self-renewing or induce differentiation in mouse embryonic stem cells [14]. In addition to studying other development systems such as hematopoietic stem cells, this technology could be used to analyze transcription response to therapeutic or signalling agents, revealing dose-response thresholds and incomplete response [15].

The finding that the SNV mutation of SP1 was only present in 20% of cells in a primary breast cancer sample shown to be 89% tumor cells by immunostaining deserves further attention. Including SP1, deep sequencing of the metastasis revealed 32 somatic point mutations [6]. By analyzing which SNVs are mutually or independently present in single cells, the mutational progression of the tumor can be reconstructed [16].

The development of this technology is well timed, as a number of strategies have recently been reported for single cell analysis of transcription. These include hybridization techniques such as mRNA-FISH [17] and rolling circle amplification [18], qPCR measurements in macroscopic volumes [19], and methods for inferring gene expression heterogeneity among small numbers of cells from laser-capture microdissection [20]. The advantages of the system presented in this thesis include higher throughput, improved specificity, large dynamic range, very low reagent cost, and simplicity. These advantages beg the implementation of this technology in a commercial product. In particular, the use of microfluidic devices for single cell sample processing as a front-end for high-throughput sequencing will enable routine single cell genomics. The microfluidic single cell RT-qPCR system developed in this work is widely applicable to biomedical research, potentially leading to single cell diagnostic assays.
References


Appendix A

Protocols

A.1 Fabrication

After fabricating the molds described in the methods section of chapter 2, multi-layer soft lithography is used to create microfluidic devices. This protocol uses polydimethylsiloxane (PDMS): specifically RTV615. The device uses a ‘push-up’ geometry, with the valve/control channels in the layer underneath the flow channels (for sample and reagents).

Before coating with PDMS, treat wafers with TMCS (trichloromethylsilane). Seal wafers in a box containing a beaker of TMCS. TMCS is volatile and will coat wafer to prevent PDMS from sticking to the photoresist (features).

A.1.1 Flow Layer

1. Mix Flow layer (thick) (5:1 A:B). Use 50 g A and 10 g B per wafer.

2. Pour 5:1 mixture on the top layer wafer in foiled dish.

3. Use pipette tips with bigger side down to centre flow wafer and push it down to release bubbles from under wafer

4. Degas the mixture/wafer by placing in vacuum for minimum 1 hour.

A.1.2 Control and Blank Layer(s)

1. While degassing, mix Control and blank layers (thin) (20:1 A:B). Use 20g A and 1g B per wafer (40 g A and 2 g B for both control and blank if being used)

2. Place control wafer on spinner and pour 20:1 mixture on about 2/3 of wafer.

3. Spin cycle:
A.1. Fabrication

- Ramp 10s to 500 rpm, hold 5s
- Ramp 5s to 1800 rpm, hold 60s
- Ramp 5s to 0 rpm

4. When finished spinning, place in a dish and close lid (can put wafer on lid because its flatter)

5. Repeat for blank(s) and then set aside blank layer.

A.1.3 Bake Flow and Control Layers

1. When there are only a few bubbles left on flow layer, remove from vacuum chamber, use pipette tip to drag bubbles off the features, and push wafer to bottom.

2. Bake Flow Layer 80°C, 60 Min.

3. Wait 15 min and then bake Control Layer 80°C, 45 Min (this way you can remove both layers from oven at the same time)

A.1.4 Align Flow Layer to Control Layer

1. Remove both layers from oven and allow to cool for a couple minutes

2. Cut on the inside of the flow wafer for a clean lift off. Cut multiple times to ensure there is no debris that might fold under flow layer and prevent proper bonding to control layer. Peel flow layer off of wafer.

3. Immediately after peeling flow layer, place it on top of control layer to minimize chance of debris getting between the 2 layers.

4. Align flow layer to control layer (still on wafer).

5. Use tape (scotch) to clean off top of the chip

6. Bake Flow and Control layers for 1 hour at 80°C.
A.1.5 Ports

1. Peel combined flow and control layers off of wafer. Make multiple cuts to ensure a clean lift off.
2. Punch holes in devices (bonded Control and Flow layers).
3. When you think you are 45 min away from finishing punching holes, bake blank layer for 45 min at 80°C
4. Clean bottom of bonded layers vigorously with tape. Remove blank layer from oven and place bonded layers control and flow layers onto the blank layer. Be sure the control (thin) layer of the bonded layers is down. Check for collapsed valves and use syringe to suck them out if necessary. Try to avoid bubbles. Clean top surface with tape.
5. Cook overnight at 80°C. Minimum 3 hours.

A.1.6 Mounting Individual Devices

1. Dice Chips
2. Place chips on glass slides. Be sure to clean slides with water and IPA first. Clean chips with tape (bottom before fastening to slide, top after fastening). Make sure plug holes are up (blank and thin on the bottom, thick on top)
3. Bake slides with chips at 80°C over night. Be sure to label the slides with information about chip, fabrication date, etc.

A.1.7 General Considerations

1. RTV stands for room temperature vulcanization. Do not let RTC A:B sit for too long (greater than 4 hours).
2. RTV A : RTV B, 10:1 is the stoichiometrically equivalent ratio.
3. Use Nitrile gloves since Latex gloves contain sulfur that may react with Pt catalyst in RTV.
4. Check that layers have baked properly before alignment (touch edge of wafers with a tweezer.)
A.2 Device Operation

The first device operation protocol presented is for a heat lysis, followed by a 2-step RT-qPCR with separate reverse transcription (RT) and qPCR steps. This protocol was used with miRNA and mRNA assays. Alternatively, a chemical lysis followed by 1-step RT-qPCR protocol is also presented. This protocol is faster, however did not work for miRNA assays, and was applied to measurements of GAPDH and SNV measurements. Krytox oil is used as the fluid in the control lines.

A.2.1 Cell Loading, Washing, and Heat Lysis

1. Device is primed with PBS containing 0.5 mg/mL BSA and 0.5 U/µL RNase Inhibitor. The bovine serum albumin (BSA) prevents cells from sticking to channel walls.

2. Cells loaded into device suspended in culture media (directly from culture). Optional off-chip wash. Cell suspensions may be drawn into microcapilllery pipette tips, and plugged into the sample inlet ports. The pipette tip is released from the pipette and air pressure is applied to opening. We used PDMS plugs to seal the pipette tips around the applied air pressure line. Alternatively, the cell suspension may be drawn into tygon tubing with a steel pin on the end of it, which is in turn plugged into the microfluidic device. Cell loading works best at concentrations between 5x10^5 and 1x10^6 cells/mL. Lower concentrations will work but it will take longer to achieve high occupancy of trapped single cells. Higher concentrations may lead to clogging in the inlet port or at the traps. Load cells at approximately 2 psi. Optionally, the peristaltic pump may be used for gentler and controlled cell loading.

3. After loading cells, perform on-chip wash to remove untrapped cells and extracellular RNA. Cells are washed with the same solution that primes the device.

4. Close valves to partition cell loading channel in order to isolate cells in capture chambers.

5. Using microscope, confirm and count which chambers contain cells (enter into spreadsheet).
6. Acutate valves to isolate cell capture chamber. Place device on flatbed thermocycler for heat lysis at 85°C for 7 minutes; followed by 4°C hold.

### A.2.2 Reverse Transcription

1. Using the ABI High Capacity Reverse Transcription kit, the RT solution prepared as below (modified from ABI protocol).

   - 10x RT Buffer: 2.00 µL
   - 5x RT primer: 4.00 µL
   - dNTPs: 1.00 µL
   - Multiscribe RT Enzyme: 1.34 µL
   - RNase Inhibitor: 0.26 µL
   - Tween 20 (1%): 2.0 µL
   - H2O: 9.4 µL

2. Reverse transcription mix is loaded into the device and flushed through the reagent injection channels.

3. RT reagent is injected into the reaction by opening the valve connecting the cell chamber to the RT chamber, and the valve connecting the cell chamber to the reagent injection line. RT chamber is dead-end filled, and then the reagent the connection to the reagent injection line is closed.

4. The device is placed on a flatbed thermocycler for a pulsed temperature RT protocol.

   - 16°C x 2 min
   - 60 cycles of (20°C x 30 s, 42°C x 30 s, 50°C x 1 s)
   - 85°C x 5 min
   - Hold 4°C

### A.2.3 Real-Time Polymerase Chain Reaction

1. The reagent mix for the PCR reaction is prepared using the ABI Taqman Universal Master Mix.
A.2. Device Operation

- 2x Taqman Universal Master Mix (ABI): 25.0 µL
- 20x Real-Time Primer/Probe: 2.50 µL
- Tween 20 (1%): 5.0 µL
- Water: 7.5 µL
- RT product (already in device): 10.0 µL

2. Flush reagent injection lines prior to injecting PCR reagent into reaction chambers (similar to RT injection). Input pressure may be increased to increase dead-end filling rate. Pressure should not be decreased as this may result in back-flow from the reaction chambers, and could lead to cross-contamination.

3. Once the PCR reaction chamber is filled, the valves closing the PCR chambers are actuated. The rest of the control lines may be removed (cut away or unplugged) to facilitate placing the device into the custom flatbed thermocycler apparatus for imaging the qPCR reaction.

4. The thermocycler controls temperature cycling for the PCR protocol
   - 95°C Hot Start for 10 mins
   - 50 cycles of 95°C x 15 s (denature) and 60°C x 1 min (anneal/extend)

A.2.4 Chemical Lysis and One-Step RT-qPCR

The chemical lysis followed by one-step RT-qPCR protocol is based on the Invitrogen CellsDirect One-Step RT-qPCR Kit.

1. For device priming and cell loading, follow steps 1-5 from the heat lysis protocol.

2. The lysis buffer is prepared following the CellsDirect kit instructions.
   - Resuspension buffer: 30 µL
   - Lysis enhancer solution: 3 µL

3. The lysis buffer is injected into the 10 nL chambers used for reverse transcription in the other protocol. This follows the same procedure of flushing the reagent injection lines prior to opening valves to permit dead-end filling.

4. Incubate lysis reaction for 10 minutes at room temperature.
5. Place device on flatbed thermocycler for heat inactivation of lysis reagent, 10 minutes at 75°C.

6. Prepare RT-qPCR reagent as instructed in CellsDirect kit, with addition of Tween 20 surfactant.
   - SuperScript III RT/Platinum Taq Mix: 1.0 µL
   - 2X Reaction Mix (with ROX Reference Dye): 25 µL
   - 20X Taqman Primers/Probes 2.5 µL
   - Magnesium Sulphate: 1.0 µL
   - Tween (1%): 5.0 µL
   - Water: 5.5 µL
   - Lysate (already in device): 10 µL (equivalent)

7. RT-qPCR reagent is injected into device similar to final PCR steps in the heat lysis protocol presented above, and the device is placed in the custom flatbed thermocycler and imaging apparatus.

8. A one-step RT-qPCR reaction is performed by RT followed by qPCR without interruption or addition of reagents.
   - 50°C for 15 minutes
   - 95°C for 2 minutes
   - 40-50 cycles of 95°C x 15 seconds and 60°C x 30 seconds
Appendix B

Experimental Setup and Design

B.1 Apparatus

The microfluidic device is controlled by pneumatic valves. These valves may be operated manually using a simple manifold, however in the current study this control was semi-automated. Solenoid actuators (Fluidigm Corp., San Francisco) are controlled through a digital input output card (NI-DAQ, DIO-32H, National Instruments), and operated using LabView (National Instruments) drivers and a custom program.

The microfluidic device is placed on a microscope stage during cell and reagent loading to facilitate visual inspection (Figure B.1A). The microfluidic device can be moved to a flatbed thermocycler for temperature control while maintaining pressure to the pneumatic valves (Figure B.1B). For qPCR the device is placed inside a custom enclosure (Fluidigm Corp., San Francisco) on a flatbed thermocycler (Figure B.2). The chuck of the thermocycler pulls a vacuum on the glass bottom of the microfluidic device for good thermal contact. The enclosure contains a mercury lamp, a CCD camera, and excitation/emission filters for fluorescence imaging. During qPCR, only one valve needs to be actuated to close all qPCR reaction chambers.

B.2 Choice of Design

The microfluidic device for single cell RT-qPCR was designed to take advantage of well-established PCR reagents and protocols. Compatibility with common and commercially available reagents also facilitates adoption of the technology in applications already using PCR methods.

The protocols for commercial PCR kits influenced the design of the microfluidic device. The microfluidic device was designed to be robust and generally applicable to short and long transcripts. In order to assay miRNA transcripts, we chose to use the recently developed miRNA RT-qPCR products available from Applied Biosystems (ABI). This protocol was also compatible with mRNA transcripts. Specifically, we
B.2. Choice of Design

used the ABI High-Capacity Reverse Transcription Kit, and the TaqMan Universal PCR Master Mix. This protocol is for a two-step, one-pot, RT-qPCR reaction. In the first step, RT reagent is added to template (cell lysate) and the RT reaction is carried out. Second, the qPCR reagent is added to the RT product and the qPCR reaction proceeds. ‘One-pot refers to the serial addition of reagents to the reaction container, with nothing taken away or purified. We implemented this two-step, one-pot, fluid handling into the architecture of the microfluidic system by serially connecting a cell capture/lysis chamber with a RT chamber and PCR chamber of increasing volume.

Determining the volumes of the reaction chambers in the device was also influenced by the commercial protocols. The RT reaction could be made up of up to 50% RNA template solution, and up to 25% of the PCR reaction could be RT product.
These dilution ratios were factored into the microfluidic chamber volumes for the RT and qPCR reactions. In an early prototype, a 1 nL cell capture/lysis chamber was connected to a 2.5 nL RT chamber (3.5 nL combined volume), followed by a 14 nL qPCR chamber. Although the dilution factors were determined by the RT kit protocol, the total volumes of the chambers were minimized in order to achieve a higher density of reactions in the microfluidic array, and therefore greater throughput. The volumes in this implementation worked for reactions involving purified RNA, but was not robust or quantitative using cells or cell lysate. By testing different concentrations of cell lysate, we determined that at high concentrations cell lysate was inhibiting the reaction. This was not surprising as the RT kit is optimized for purified RNA. We explored the use of oligo-dT beads for purifying RNA in the device prior to performing reactions. This work is ongoing, however the use of beads required additional microfluidic complexities (such as sieve valves for stacking purification columns, or magnets for immobilization), and the surface chemistry of the beads would need to be modified in order to capture miRNAs.
B.3. Design Considerations

Taking advantage of the rapid prototyping in multilayer soft lithography, we designed a microfluidic device to test different volumes and different dilution factors for RT and qPCR reactions starting from cells or different cell lysate concentrations. This device confirmed our earlier observation that cell lysate inhibits RT-qPCR reactions at high concentrations, and revealed volumes that would reliably work in the dynamic range expected for single cells. This resulted in the design presented in this thesis, consisting of a 0.6 nL cell capture/lysis chamber, connected to a 10 nL RT chamber, and a 50 nL qPCR chamber.

During the development of this larger volume device we also explored alternative commercial products for performing RT-qPCR direct from cells. ABI offers the Cells-to-CT kit, which adds a chemical lysis step prior to using the High Capacity cDNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix. Implementing this chemical lysis would have required two additional chambers: one for injecting the lysis buffer, and one for adding a lysis neutralizing agent, before connecting to the RT and qPCR chambers. Furthermore, the volumes and dilutions expected in the protocol resulted in large chambers in the microfluidic device, limiting density of integration. In designing the final version of the device, we were also interested in the CellsDirect RT-qPCR kit from Invitrogen as it was optimized for working with cells without lysate inhibition. The CellsDirect RT-qPCR kit uses a chemical lysis agent that is neutralized by heat, followed in a one-pot fashion by a one-step RT-qPCR where the reagents and enzymes for RT and qPCR are combined in a single reaction vessel, and the RT reaction is followed by qPCR without interruption or addition. The fluid processing for this protocol required the serial injection of two reagents in increasing volume (lysis buffer, then RT-qPCR mix), and is similar to the ABI 2-step RT-qPCR fluidic requirements (lysis by heat, reagent injections of RT mix, then qPCR mix). The volumes in the final device (10 nL RT chamber, 50 nL PCR chamber) were chosen to accommodate both the Invitrogen CellsDirect protocol and the ABI High Capacity cDNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix.

B.3 Design Considerations

A number of design considerations arose from working with polydimethylsiloxane (PDMS). PDMS is gas permeable, and this property is useful for allowing the dead-
end filling of chambers. However, this gas permeability results in evaporation from within the device. This evaporation can effect reactions, altering concentrations. By incorporating a dense array of sufficiently large chambers, we found this evaporative effect to be negligible. We had difficulty performing reactions with SYBR green, an intercalating dye. Although we were able to measure qPCR amplification and perform melting curve analysis, the signal was very weak, and the signal-to-noise ratio was low. This is likely due to the small size of the SYBR green molecules, allowing them to be absorbed into the PDMS. This was one of the factors in selecting TaqMan probe assays.

Integrated into the microfluidic device is a peristaltic pump, comprised of three valves. This pump is downstream of the cell loading (so it does not mechanically damage cells passing through) and reagent injection lines. The pump permits precise control over fluid flow rates, and was integrated in order to gently manipulate cells. However, we found that the cell traps and reactions worked well in both pumping and pressure-driven flow regimes, and the latter was significantly faster.

The reagent injection lines are addressed through a large fluidic bus. This fluidic bus has a low fluid flow impedance compared to the reagent injection lines, resulting in (approximately) even flow each line. Using a fluidic bus has advantages over bifurcating tree structures as any number of lines can extend from the bus (rather than only numbers satisfying $2^n$).