A microfluidic platform for quantitative analysis of single mycobacteria cells

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Dissertation

A MICROFLUIDIC PLATFORM FOR QUANTITATIVE ANALYSIS
OF SINGLE MYCOBACTERIA CELLS

by

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ABSTRACT

*Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis (TB), is
the leading bacterial cause of death worldwide. A significant barrier to global MTB
eradication is 'latent' TB infection, where MTB persists in the human host in a
metabolically dormant and highly drug-tolerant state. Latently infected individuals
constitute a vast global reservoir of disease (~2 billion people worldwide), and the
heightened drug tolerance of dormant MTB necessitates long antibiotic treatments (up to
9 months of combination antibiotic therapy).

MTB dormancy is thought to be the result of an adaptive response to host-induced
stresses, involving coordinated transcriptional regulation of hundreds of genes as well as
numerous metabolic changes. Currently, our understanding of this process is limited by a
lack of tools for studying dynamic behavior in single cells. Gene regulation is a dynamic
phenomenon that occurs within each cell individually, but many assays rely on steady-
state measurements of a population average and thus fail to capture important information
about the dynamics of cellular behavior. Additionally, cell-to-cell phenotypic variation
has been identified as a key source of microbial drug tolerance, further highlighting the need for single-cell studies.

To address this need, we developed a microfluidic platform to study *Mycobacteria* species at the single-cell level. This platform enables on-chip culture and fluorescent imaging of live cells in precisely controlled conditions, and can thus be used to study dynamic processes within single cells as well as phenotypic heterogeneity across a cellular population. We used this platform to obtain diverse new insights about mycobacterial biology, using the fast-growing mycobacterium *M. smegmatis*. 1) We directly observed gene regulation by the transcription factor KstR in single cells, confirming regulatory interactions that had been predicted computationally. 2) We analyzed morphology, growth, and division data across hundreds of single cells and found that cell division in *Mycobacteria* is governed using size-based, rather than time-based, control mechanisms. 3) We found that individual cells exhibit considerable differences in their responses to antibiotic stress, and that these differences have implications for cellular survival.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................ iv
ABSTRACT ......................................................................................... vi
TABLE OF CONTENTS ........................................................................ viii
LIST OF TABLES ................................................................................ xi
LIST OF FIGURES .............................................................................. xii
LIST OF ABBREVIATIONS ................................................................... xvi
GLOSSARY ........................................................................................... xvii

CHAPTER ONE: BACKGROUND ............................................................. 1
Section One: Biology and Public Health Significance of \textit{M. Tuberculosis} .......... 1
Subsection One: Tuberculosis is a Major Global Health Burden ..................... 1
Subsection Two: MTB Persistence, Dormancy, and Drug Tolerance ............... 5
Subsection Three: Insights from the MTB Regulatory Network ....................... 7
Section Two: Quantitative, Real-Time Measurements of Single Cells .......... 9
Subsection One: Phenotypic Heterogeneity In Single Cells ......................... 9
Subsection Two: Dynamic Processes in Single Cells .................................... 11

CHAPTER TWO: A Microfluidic Platform for Live-Cell Imaging of \textit{Mycobacteria} .... 14
Section One: Microfluidic Device Design and Fabrication .......................... 14
Subsection One: Microfabrication in SU-8 and Silicon ................................. 15
Subsection Two: PDMS Soft Lithography ............................................... 18
Section Two: Device Performance and Optimization ................................................. 20
Subsection One: Cell Loading and Trapping .......................................................... 21
Subsection Two: Live-Cell Imaging ......................................................................... 27
Section Three: Image Segmentation and Analysis .................................................. 29
Subsection One: Image Processing with Miji ......................................................... 30
Subsection Two: MicrobeTracker ............................................................................ 31
Subsection Three: MATLAB Processing/Analysis .................................................. 32
Section Four: Molecular Cloning in *M. smegmatis* ............................................. 35
Subsection One: Description of Protocols .............................................................. 35
Subsection Two: Inducible Promoter Systems and Fluorescent Protein Constructs ... 36
Subsection Three: List of Strains ........................................................................... 39

CHAPTER THREE: Quantitative Time-Lapse Imaging of *M. smegmatis* Cells ...... 42
Section One: Quantifying KstR’s Regulation of Predicted Target Genes ............... 43
Subsection One: Tet-Inducible KstR ...................................................................... 43
Subsection Two: Oscillator-Driven KstR ............................................................... 45
Section Two: Morphology and Growth Measurements in Single Cells .................. 52

CHAPTER FOUR: Behavior of *M. smegmatis* in Response to Diverse Stresses ....... 59
Section One: HspX Expression and Cell Fate Under Antibiotic Stress .................... 59
Section Two: Cellular Responses to Hypoxia ....................................................... 73

CHAPTER FIVE: Conclusions and Future Directions .............................................. 76
Section One: DosR Expression in Response to Hypoxia and Other Stresses .......... 77
Section Two: Further Investigation of the MTB Regulatory Network .................... 78
LIST OF TABLES

Table 1. Mycobacterial strains and plasmids used in this work.............................. 41
LIST OF FIGURES

Figure 1.1. TB incidence in the United States and worldwide. Adapted from [1] .............2

Figure 1.2. The two major patterns of MTB infection. .................................................4

Figure 1.3. ChIP-Seq based ‘hairball’ model of the *M. tuberculosis* transcriptional regulatory network. TFs and genes are represented as circles and regulatory interactions are represented as lines. ..............................................................7

Figure 2.1. Process for making PDMS microfluidic devices from an SU-8 mold. ...........16

Figure 2.3. PDMS microfluidic device on a glass coverslip with channel architecture and inlet holes clearly visible.................................................................20

Figure 2.4. Architecture of the earliest microfluidic device used in these experiments, adapted from [59]. .................................................................22

Figure 2.5 Rhodamine dye was flowed into the cell trapping region for 60 seconds and replaced with 7H9 media. .......................................................23

Figure 2.6 *M. smegmatis* cells in the trapping channel of the microfluidic device. This trap contains approximately 15 cells, whereas >30 cells are preferred for imaging experiments. .................................................................24

Figure 2.7 The CellAsic-style cell trapping device resulted in improved cell trapping efficiency.................................................................26

Figure 2.8 Screenshot of the NIS-Elements user environment during a typical timelapse experiment.................................................................28

Figure 2.9. Screenshot of MicrobeTracker program. Cell 23 is marked by the black arrow. .................................................................32
Figure 2.10 Screenshot showing output of DrawGrowthRate.m .................................................. 34

Figure 2.11. Representative plate-reader and microfluidics experiments showing repeated transcriptional activation of Tet-KstR-GFP in response to ATC addition (50 ng/mL). .................................................................................................................. 38

Figure 2.12. Schematic of a PMV306 integrating vector used in this work .................. 40

Figure 3.1. Movie montages showing expression of Tet-inducible KstR_GFP and MS6038p_RFP after addition of ATC. As KstR is produced, expression of the KstR target MS6038 is repressed .......................................................... 45

Figure 3.2. Schematic showing architecture of the synthetic oscillator circuit .......... 46

Figure 3.3. Computational prediction of gene expression from the oscillator circuit, and experimentally obtained single-cell fluorescent traces from cells containing the circuit. Inducer concentrations: 25 ng/ml aTe, 14 mM Cap .................................................. 47

Figure 3.4. Representative image series of oscillatory KstR-RFP expression in single cells ........................................................................................................................................ 48

Figure 3.5. Representative single-cell traces of KstR-RFP and d(MS1953-YFP)/dt ........ 50

Figure 3.6. Cross-correlations show regulatory activity of KstR on two of its targets .......................................................................................................................... 51

Figure 3.7. Image series of single M. smegmatis cells showing cell segmentation and numbering, as well as the characteristic V-shape of newly divided cells .................................................................................................................. 54

Figure 3.8. Lineage trace diagram for a cell (“Cell 4”) in an M. smegmatis microcolony, and single-cell size measurements for the same microcolony .................................................................................................................. 55
Figure 3.9. Growth and division characteristics in *M. smegmatis*. Cell size at birth versus (a) growth rate and (b) doubling time. (c) Doubling time versus growth rate. (d) Cell size at birth versus at division.

Figure 4.1. Induction of *HspX* at low oxygen tension. Adapted from [26].

Figure 4.2. Predicted transcriptional regulators of *HspX*. Taken from tbdb.org.

Figure 4.3. Single-cell ECFP fluorescence traces in response to 30 ug/mL D-Cycloserine.

Figure 4.4. Image frames showing single *M. smegmatis* cells expressing HspXp:ECFP in response to 30 ug/mL D-Cycloserine.

Figure 4.5. Single-cell ECFP fluorescence traces in response to 100 ug/mL D-Cycloserine.

Figure 4.6. Single-cell ECFP fluorescence traces in response to 30 ug/mL DCS.

Figure 4.7. Single-cell length measurements under DCS treatment (30 ug/mL).

Figure 4.8. Single-cell length measurements under DCS treatment (100 ug/mL).

Figure 4.9. Single-cell growth rates, DCS 30 ug/mL. Green lines, single cells; black line, population average.

Figure 4.10. Single-cell growth rates, DCS 100 ug/mL. Green lines, single cells; black line, population average.

Figure 4.11. Odds of cellular survival vs. number of HspXp-ECFP fluorescence peaks.

Figure 4.12. Single-cell growth rates pre- and post-addition of 30 ug/mL DCS. Red histograms, non-surviving cells. Green histograms, persister cells.
Figure 4.13. Single-cell growth rates pre- and post-addition of 100 ug/mL DCS. Red histograms, non-surviving cells. Green histograms, persister cells...

Figure 4.14. Growth rates pre-drug addition for pulsing vs. non-pulsing cells.

Figure 4.15. Growth rates post-drug addition for pulsing vs. non-pulsing cells.

Figure 4.16. Ruthenium fluorescence within a microfluidic 'hypoxia chamber.'

Figure 4.17. Induction of the nar promoter in *E. coli* in response to hypoxic conditions in a microfluidic device. Oxygen displacement began at time t=0. Time in minutes...
LIST OF ABBREVIATIONS

CO .................................................................................................... Carbon monoxide
ECFP ................................................................................................... Enhanced cyan fluorescent protein
GFP ..................................................................................................... Green fluorescent protein
kB ...................................................................................................... kilobase, i.e. 1,000 nucleotides
MDR-TB .......................................................................................... Multiply drug-resistant *M. tuberculosis*
MTB .............................................................................................. *Mycobacterium tuberculosis*
NO ................................................................................................... Nitric oxide
PDMS ............................................................................................... Poly(dimethylsiloxane)
RFP ................................................................................................... Red fluorescent protein
TB ...................................................................................................... Tuberculosis (the disease)
XDR-TB .......................................................................................... Extensively drug-resistant *M. tuberculosis*
GLOSSARY

Antibiotic tolerance: A transient, phenotypic decrease in drug susceptibility. This is different than antibiotic resistance, which refers to a genetic mutation.

Dormancy: A non-replicating or slowly-replicating state of MTB thought to allow the organism to survive in the host for long time periods.

Latent MTB: Clinically asymptomatic MTB infection. Latent MTB describes a disease, and does not refer per se to the MTB dormancy phenotype.

Persisters: Antibiotic-tolerant cells. These are often observed as a subpopulation of a larger, drug-sensitive colony and are a major cause of refractoriness in bacterial infections. Several diverse mechanisms of persister formation have been reported, from slow growth to the overexpression of “persistence genes”.
CHAPTER ONE: BACKGROUND

In this Chapter the scientific and public health context of this dissertation work is described. Section One focuses on the public health threat posed by \textit{M. tuberculosis} (MTB) and in particular, the challenges of treating latent tuberculosis (TB) infections. Current scientific opinion regarding MTB dormancy and drug tolerance is summarized, including recent insights from the MTB gene regulatory network. Section Two introduces recent techniques for acquiring quantitative measurements of single cells in real-time and discusses the advantages of single-cell techniques. These advantages include the ability to observe heterogeneity within a population of cells, and to measure the dynamics of cellular processes.

Section One: Biology and Public Health Significance of \textit{M. Tuberculosis}

Tuberculosis has been known to mankind since antiquity, and yet it confounds attempts at global eradication. The reasons for its continued success as a human pathogen are multifaceted and still not completely understood. This Section describes the global health burden of MTB, with a particular focus on the phenomenon of MTB dormancy and drug tolerance, and summarizes current scientific opinion regarding TB pathogenesis.

Subsection One: Tuberculosis is a Major Global Health Burden

The pathogenic organism \textit{Mycobacterium tuberculosis} (MTB) is the causative agent of the infectious disease tuberculosis (TB) and is the leading bacterial cause of
death in humans [1]. Indeed, the scope of the TB epidemic has been called a “global health emergency” [2]. MTB is responsible for three human deaths every 60 seconds, and in 2010 there were an estimated 8.8 million new cases of TB and 1.5 million TB-associated deaths [3]. The prevalence of TB in the United States has declined dramatically since 1992 [4], to a rate of 3.2 cases per 100,000 persons (Figure 1.1). However, TB incidence in developing countries and resource-limited areas is often 10 to 100 times higher than in the United States, with incidence rates in some countries reaching >1,000 cases per 100,000 persons [1].

Figure 1.1. TB incidence in the United States and worldwide. Adapted from [1]
A main cause of MTB's success as a human pathogen is its ability to reside in the human host for years or decades in a dormant state. This dormancy results in a so-called 'latent' state of infection, i.e., one that does not present discernible external symptoms in the infected host [5, 6]. In latent TB infection, the organism persists in a metabolically dormant state within alveolar macrophages. [7] Heightened drug tolerance of MTB in the dormant state has been identified as a key barrier to eradication of TB worldwide [8], and necessitates long antibiotic treatments (up to 9 months of combination antibiotic therapy) [9]. Long antibiotic treatment regimens are expensive and difficult to carry out to completion, especially in low-resource settings, and incomplete antibiotic treatment has been identified as a major factor in the rise of drug-resistant tuberculosis strains (multidrug-resistant tuberculosis, MDR-TB, and extensively drug-resistant tuberculosis, XDR-TB) [10]. Latently infected individuals constitute a vast global reservoir of MTB, further hindering efforts at eradication; it is estimated that fully one-third of the world's population (> 2 billion people) harbor latent TB infection [1].

An overview of the process by which MTB infects humans is diagrammed below in Figure 1.2, adapted from [11]. TB is spread via inhalation of MTB bacilli that are released into the air (e.g. via coughing) by an infected person. Infection begins with exponential growth of MTB within macrophages; clusters of macrophages then begin to aggregate and form a characteristic lesion known as a granuloma [12]. MTB is able to evade mechanisms of innate and adaptive immunity [13] using strategies that are not fully understood but that include disruption of native host processes such as phagosome maturation and macrophage apoptosis [14-16]. 90-95% of infections result in MTB's
dormancy adaptation response and its transition to nonreplicating persistence, which is thought to occur at 2-4 weeks post-infection. Inaccessibility of the interior of the granuloma to antibiotic drugs is thought to contribute to drug tolerance of MTB in latent infection. As described below, reactivation of latent TB infection can occur when the individual becomes immune-compromised due to illness or if the immune system is otherwise weakened. Active TB, either from a newly acquired infection or from reactivation of a latent infection, results in contagious spread of the pathogen to new human hosts.

Figure 1.2. The two major patterns of MTB infection.
An especially devastating problem associated with MTB in developing countries is the co-incidence of TB and HIV infection [17]. TB is the leading killer of HIV-positive individuals worldwide, accounting for approximately 23% of HIV-related deaths [18]. The weakened immune system of HIV-positive individuals is a key cause of increased co-morbidity. In individuals with latent TB infection who have healthy immune systems, there is a 10% chance that latent TB will “reactivate” and progress to active infection at some point during the individual’s lifetime. However, in immune-compromised (e.g. HIV-positive) individuals the probability of reactivation increases to 10% per year [17].

To add to these alarming statistics is the recent emergence of drug-resistant strains of MTB, which have evolved mechanisms of genetic antibiotic resistance [10]. These are designated multidrug-resistant tuberculosis (MDR-TB), which refers to MTB strains resistant to the two most common anti-tuberculosis drugs (isoniazid, INH, and rifampicin, RIF), and extensively drug-resistant tuberculosis (XDR-TB), which refers to strains that are resistant to INH and RIF as well as second-line antibiotics (e.g. kanamycin) and fluoroquinolones [1]. Incomplete treatment of latent TB infections has been identified as a major factor underlying the emergence of drug-resistant MTB strains, and further underscores the need to understand mechanisms of phenotypic drug tolerance in MTB pathogenesis.

**Subsection Two: MTB Persistence, Dormancy, and Drug Tolerance**

MTB’s ability to enter a state of non-replicating persistence is a major cause of its success as a human pathogen [9]. This phenomenon is thought to be an adaptive response
of the organism to host-mediated stresses, which include hypoxia (low oxygen availability), nutrient starvation, high carbon monoxide (CO) and nitric oxide (NO) concentration, and acidic pH [19-23]. These environmental cues cause a dramatic response in MTB that involves coordinated transcriptional regulation of hundreds of genes as well as numerous metabolic changes [24]. This response is at least partly mediated by the transcriptional regulator DosR (Rv3133c), which has been termed the "master regulator" of dormancy and which controls a regulon of ~50 MTB genes [25, 26]. DosR, whose name in fact stands for "Dormancy survival regulator," was shown to be critical for viability of MTB cultures grown in in vitro hypoxia models, and loss of viability in DosR knockout strains of MTB was shown to coincide with loss of induction of DosR regulon genes [27, 28]. The gene HspX (Rv2031c), also known as acr (alpha-crystallin), is the most strongly-induced gene in the DosR regulon and has been studied as a surrogate, or indicator, of the DosR regulon in general [26, 29, 30]. The work described in Chapter Four of this dissertation measures HspX transcription in response to stresses. While DosR has been thought of as the primary regulator of the hypoxic response in MTB, in fact nearly one-third of all MTB genes exhibit transcriptional changes in response to hypoxia [24], and a 2009 study found that DosR regulon induction was shown to be inessential for drug tolerance under hypoxic conditions in vivo and in vitro [31]. These findings suggest that our understanding of MTB's adaptations to host-induced stresses, and the state of the organism during non-replicating persistence and dormancy, are incomplete and require further investigation. A better understanding of how MTB dormancy in the host specifically relates to drug tolerance would help guide
the development of drugs targeted to dormant MTB, which could enable shortened antibiotic treatment regimens for active and latent TB infection.

**Subsection Three: Insights from the MTB Regulatory Network**

The regulatory mechanisms underlying MTB's adaptive response to host-induced stresses are still unknown, largely because we lack a comprehensive understanding of the MTB regulatory network. As such, only a small fraction of the >180 MTB transcription factors (TFs) have validated functionalities, and the interactions between TFs – necessary for the regulation of complex behavior – have not been studied. To address these issues, the Galagan lab recently used ChIP-seq based techniques to construct the first genome-scale map of the *M. tuberculosis* regulatory network (Figure 1.3) [24].

![Figure 1.3. ChIP-Seq based 'hairball' model of the *M. tuberculosis* transcriptional regulatory network. TFs and genes are represented as circles and regulatory interactions are represented as lines.](image-url)
ChiP-Seq is the shorthand name for an in-vivo method of identifying genome-wide binding sites of a transcription factor, entailing Chromatin ImmunoPrecipitation followed by DNA Sequencing [32]. In this procedure, a protein of interest is crosslinked to genomic DNA, which is then sheared into fragments of about 1,000 base pairs, or 1 kilobase (kB). Because the protein preferentially binds to its sequence-specific binding sites, fragments containing these sites will contain a protein-DNA complex. Then, in the immunoprecipitation step, these complexes are filtered out using an antibody for the protein of interest. Once filtered, the DNA-protein complexes are unlinked and the DNA fragments are sequenced, showing where in the genome the protein was bound. This method allows for extremely high redundancy and enables very high resolution: in other words, it allows researchers to pinpoint with high precision all the locations in the genome that are recognized and bound by the protein. By repeating this process for many transcription factors in the network, a reconstruction of the organism’s regulatory network topology emerges. Many of the TFs identified by this work have analogous genes in *M. smegmatis*, which makes them amenable for study in biosafety level 1 (BSL-1) facilities. Intriguingly, this work [24] identified a regulatory subnetwork linking between hypoxic adaptation, cholesterol degradation, and lipid biosynthesis, through the regulators Rv0081, Rv3024, Rv3133c (DosR), and Rv3574 (KstR). Additionally, this work identifies network motifs such as feed-forward loops [33] that have been shown to underlie dynamic behavior in regulatory networks [34, 35]. The work described in Chapter Three of this dissertation was undertaken in an attempt to further explore the
predictions of this regulatory network model, and to provide direct confirmation of regulatory interactions predicted by ChIP-Seq and microarray datasets.

Section Two: Quantitative, Real-Time Measurements of Single Cells

Quantitative, real-time measurements of single cells provide dynamic information that can be helpful for elucidating mechanisms underlying cellular phenotypes and processes, because they provide information that is impossible to obtain via steady-state measurements of a bulk population average. Measuring the fluorescence intensity from a bulk flask culture, with a concentration of $10^6$-$10^9$ cells/mL, reveals nothing about the fluorescence activity of individual cells. Two specific advantages of single-cell measurements are the ability to study phenotypic heterogeneity in a population, and to obtain a clearer understanding of dynamic processes (e.g. stress adaptations, or cell division). The importance of each of these phenomena is described here using specific examples from the literature, in order to put this dissertation work in context.

Subsection One: Phenotypic Heterogeneity In Single Cells

Phenotypic variation is a widespread phenomenon in bacteria, and has been shown to play a functional role in maintaining population fitness [36]. Isogenic microbial populations exhibit considerable cell-to-cell phenotypic heterogeneity, allowing some cells to survive environmental stresses that would otherwise eradicate the entire population [37]. Heterogeneity in a population can occur as a single Gaussian distribution or as a multimodal distribution comprising subpopulations (the size of which might be
similar or quite different). This heterogeneity can arise due to stochastic fluctuations in cellular components, transcription rate differences, variations in cell age or growth stage, and regulatory architecture such as feed-forward loops that are highly sensitive to small imbalances and lead to bistability [38-40]. These mechanisms result in the formation of distinct subpopulations of bacteria within a clonal population [41, 42]. Importantly, discovering these heterogeneities is difficult via many methods (e.g. global transcriptional profiling via microarray [43]) because these assays necessarily involve taking a population-average measurement, which masks cell-to-cell variation [44]. Recent methods such as flow cytometry can sort cells into a small number of subpopulations based on fluorescence measurements, but are limited in their ability to monitor live cells [45]. Microfluidic devices, however, offer a way of quantitatively measuring differences in gene expression, cell morphology, and cellular behavior (e.g. differentiation) in live cells, and offer in vivo-like microenvironments. These devices are thus uniquely well suited for studies of phenotypic variation.

Of particular relevance to this thesis work is the finding that phenotypic heterogeneity in a microbial population is a key mechanism of persister formation [37]. Sub-populations of antibiotic-tolerant 'persister' cells have been observed in every pathogen yet investigated, including MTB, and are a major cause of refractoriness in bacterial infections [5, 6]. Importantly, the drug tolerance of persister cells is not due to a genetic change, but is rather a reversible phenotype [6]. For example, one mechanism of persister formation involves variable cell-to-cell expression of stress-response genes (e.g. HipA and RelE) [46-48]. Recent work has shown a clear link between single-cell
variation and drug tolerance in Mycobacteria species [49-51]. Aldridge et al. showed that
*M. smegmatis* cells generate cell-to-cell variability in size and growth rate; these
differences were correlated with susceptibility to antibiotics targeted to cellular growth
processes [49]. Wakamato et al. observed that *M. smegmatis* cells exhibit stochastic
'pulses' in expression of KatG, an enzyme that activates the prodrug isoniazid (INH).
These pulses resulted in cell-to-cell variation in KatG expression levels, which affected
INH-mediated cell death [50]. These studies suggest that cell-to-cell heterogeneity is
potentially an important determinant of MTB drug tolerance, and investigating
*Mycobacteria* at the level of single cells was critical for obtaining these results. Drug­
tolerant microbial persisters highlight the need to investigate single-cell phenotypic
variation as a source of population-level antibiotic tolerance.

*Subsection Two: Dynamic Processes in Single Cells*

Cells employ multiple levels of regulation to coordinate gene expression, with
regulatory network topology defining complex patterns of expression in regulated genes.
Gene expression patterns, then, are dynamic responses to transient and fluctuating
stimuli. These responses are regulated by multiple TFs, and as such are governed by
regulatory elements including feedback, delay, logic gates, and autoregulation, and
epigenetic factors such as post-transcriptional modification. To get a complete picture of
regulatory dynamics thus requires the acquisition of quantitative data from single cells
over time, rather than obtaining a population average measurement at only one timepoint.
Examples of both positive and negative feedback elements are pervasive in biology, with
diverse functional roles being ascribed for these subnetwork architectures [52, 53]. In MTB, evidence exists to support the idea that the TF DosR may in fact function as a bistable switch, as it positively autoregulates its own promoter [54, 55]. This finding has implications for the dynamics of MTB’s entry into dormancy in response to host-induced stresses, as bistable network elements naturally give rise to diverse cellular phenotypes. Additionally, functional roles for high-amplitude, low-frequency ‘pulsing’ in genetic circuits have been reported in prokaryotic and eukaryotic regulatory networks [56]. Finally, the dynamics of cellular responses to stress are most appropriately studied in single cells due to asynchronous response time in single cells, and the possibility of differential responses across a population. Microfluidic platforms for single-cell analysis of *Mycobacteria* species are uniquely well suited to study adaptations to stresses due to the capabilities of the platform for environmental control; in Chapter Five some potential future directions for this work are explored.

In summary, copious examples from diverse bacterial organisms (including MTB) and eukaryotes point to the need for quantitative and dynamic measurements of single cells in order to more accurately model dynamic processes, recapitulate predictions of computation regulatory network models, and quantify the extent and possible significance of heterogeneous phenotypes within a population of isogenic cells. Microfluidic devices offer an attractive solution for this problem because they allow live-cell imaging of individual cells over multiple cell cycles with fine control over environmental conditions. Chapter Two describes the methods used to design and
fabricate such a device for *Mycobacteria*, the first of its kind specifically designed for studies of this organism.
CHAPTER TWO: A Microfluidic Platform for Live-Cell Imaging of *Mycobacteria*

Microfluidic devices have become popular tools for systems biology studies because they enable fluorescence data to be gathered, simultaneously and independently, from many individual cells over long time periods. Researchers have recently employed these devices in conjunction with time-lapse microscopy systems to study prokaryotic and eukaryotic organisms [53, 57]. The advantages of this technique include the ability to measure and quantify cell-to-cell heterogeneity and dynamic processes, as described in Chapter 1, and exquisite environmental control [52]. In particular, several groups were able to gain novel insights into biological processes in *E. coli* using microfluidic time-lapse microscopy platforms [58-60]. However, before this thesis work began, there existed no analogous studies reporting the successful trapping and culture of *Mycobacteria* on-chip. This Chapter describes efforts to build such a device and to optimize it for use with *M. smegmatis*.

Section One: Microfluidic Device Design and Fabrication

In designing a microfluidic cell-trapping device for *Mycobacteria* species, the materials selection process was guided by previously published reports describing the successful use of poly(dimethylsiloxane) (PDMS)-based microfluidic devices bonded to glass for live-cell imaging [61-63]. Design criteria for microfluidic cell-trapping devices include the following: (1) cells must remain stationary in the x, y, and z-planes for the duration of the experiment; (2) growth conditions of the cells (such as temperature and CO2 content) must be met and maintained; (3) the device must not interfere with
fluorescent image capture; and (4) fine control over the cells’ environment, such as the ability to change the concentration of a chemical in the medium, is required for more complex experiments. The advantages of PDMS for this application include its optical transparency, gas permeability, low cost, and relative ease of prototyping. Using photolithography techniques for patterning SU-8 photoresist onto silicon, a device mold can be generated from a pattern designed in AutoCAD in <24 hours. This silicon mold may then be used to generate many copies of the PDMS device. This Section provides an overview of these device fabrication processes: namely, ‘hard lithography’ using SU-8 and silicon, and ‘soft lithography’ using PDMS and glass coverslips.

*Subsection One: Microfabrication in SU-8 and Silicon*

Microfabrication techniques including photolithography (literally “writing with light”) were originally developed by the electronics industry and have now found widespread use in biomedical research [64]. Ultraviolet (UV) photolithography involves user-defined patterning of a UV-sensitive material known as photoresist. The photoresist SU-8 exists as a viscous liquid until it is exposed to ultraviolet light, at which point it solidifies due to chemical crosslinking. By employing an opaque stencil known as a photomask to specify areas of UV crosslinking, the researcher can thus can create patterns of solid SU-8 features with micron-scale dimensions [65]. This process is typically performed on a flat silicon wafer in a Class 1000 cleanroom. The SU-8 photolithography protocol is briefly summarized below; detailed protocols are published in the SU-8 user documentation [66]. The description provided here focuses on those
steps in the protocol that are most critical for achieving the sub-micron feature heights necessary for \textit{M. smegmatis} cell trapping. An overview of the entire process is diagrammed below in Figure 2.1.

<table>
<thead>
<tr>
<th>STEP</th>
<th>3-D VIEW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deposit SU-8 on silicon wafer</td>
<td><img src="image1" alt="Diagram" /></td>
</tr>
<tr>
<td>Align mask; expose to UV</td>
<td><img src="image2" alt="Diagram" /></td>
</tr>
<tr>
<td>Develop features</td>
<td><img src="image3" alt="Diagram" /></td>
</tr>
<tr>
<td>Pour PDMS onto SU-8 mold</td>
<td><img src="image4" alt="Diagram" /></td>
</tr>
<tr>
<td>Allow PDMS to cure</td>
<td><img src="image5" alt="Diagram" /></td>
</tr>
<tr>
<td>Peel off PDMS</td>
<td><img src="image6" alt="Diagram" /></td>
</tr>
<tr>
<td>Cut out PDMS device and bond to glass</td>
<td><img src="image7" alt="Diagram" /></td>
</tr>
</tbody>
</table>

Figure 2.1. Process for making PDMS microfluidic devices from an SU-8 mold.
SU-8 polymer is sold in various formulations that differ in their viscosity, such that coating a silicon wafer with liquid SU-8 and spinning the wafer at a defined speed (e.g. 3000 rpm) will result in a layer of uniform thickness. This process is known as spin-coating, and the coating thickness depends heavily on the viscosity of the particular SU-8 formulation in use. For example, in Figure 2.2 (reproduced from [66]) it can be seen that a spin speed of 2000 RPM will produce a 2.4-micron thick coating if SU-8 2002 is used, but the same spin speed will produce a 0.6-micron thick coating of SU-8 2000.5. Typical spin times for this process step are 30 seconds.

![SU-8 2000 Spin Speed vs. Thickness](image)

**Figure 2.2.** SU-8 film thickness as a function of spin speed. Reproduced from [15].

After spin-coating, the wafer is placed on a 65°C and then a 95°C hotplate for 1-2 minutes to allow evaporation of excess solvent. The SU-8 coated wafer is then placed in a UV mask aligner (Karl Suss) and exposed to UV radiation such that ~100 mJ/cm² of
exposure energy is obtained. The precise exposure times vary from machine to machine and must be calibrated for the specific process due to such factors as bulb brightness, lifetime, and mirror cleanliness. In this work, four wafers were typically prepared each time a new device mold was being fabricated, so that adjustments to the UV exposure step could be made as necessary.

After UV exposure, a ‘post-bake’ step is performed, again comprising of 1-2 minutes of treatment on a 65°C hotplate and then 1-2 minutes on a 95°C hotplate. This step is intended to accelerate the polymerization of the SU-8, and indeed the patterned features become visibly discernible at this step. Finally, the wafer is placed in SU-8 developer solution (MicroChem) and agitated or shaken to dissolve un-crosslinked SU-8. For features in the 25-75 micron range, development may require 5-7 minutes of immersion, but for sub-micron features it is recommended to develop for only 5-10 seconds at a time in order to avoid over-development and degradation of the features. After development, a “hard bake” step may be performed, which entails 10-30 minutes at 150°C and further improves the mechanical properties of the SU-8 features. However, for submicron features to be used as a mold for PDMS devices, the hard bake is unnecessary.

**Subsection Two: PDMS Soft Lithography**

PDMS, also known as dimethicone, is a low-cost and optically transparent polymer with high gas permeability and native hydrophobicity, and as such is an attractive material for microfluidic devices for use with live cells [67, 68]. PDMS-based microfluidic devices can be fabricated using an SU-8 mold (as described in Subsection
One) as a master, via soft lithography techniques. PDMS consists of two liquid components – a polymer and a crosslinker – that solidify into an optically transparent, solid silicone rubber after mixing. This process is accelerated at high temperatures; therefore, when liquid PDMS is poured onto the SU-8 mold and baked at 80°C, solid devices can be peeled off the wafer in less than two hours, meaning that multiple ‘batches’ of PDMS devices can be fabricated per day from a single SU-8 mold. This transfer process has high fidelity in the feature size range applicable to this project (i.e. 0.9 micron feature height, and 10-100 micron feature width); that is, wherever there were raised SU-8 features on the silicon wafer, analogous ‘grooves’ will be present in the PDMS. After curing, holes are punched through the device where access tubes will be needed during experiments; then, the patterned PDMS and a clean glass coverslip are bonded together using an oxygen plasma bonder. The plasma bonder creates free radicals in the exposed siloxane and silicon dioxide (glass) surfaces, which are then pressed together to form covalent bonds [69, 70]. This creates a watertight network of PDMS microchannels, accessible via the inlet holes, bonded to the glass coverslip (Figure 2.3 below).

The power of using SU-8 and PDMS-based photolithography lies in its low cost and high throughput, allowing for very rapid prototyping of new device designs. The entire fabrication process, from AutoCAD design to functional PDMS device, can theoretically be performed in less than 48 hours. In addition, small device design changes (i.e. modifying the channel height) are performed at the SU-8 level and thus do not require a new photomask to be designed or fabricated, further simplifying the prototyping
process. A criticism of PDMS-based microfluidic devices is that their material properties render them unsuitable for field-based devices such as point of care diagnostics [71], and as such these devices are appropriate only for laboratory settings. For single-cell time-lapse imaging experiments such as the work described in this thesis, however, PDMS-and-glass based devices are entirely appropriate, and indeed the ease and low cost of these devices have made them a true enabling technology for single-cell biology studies.

Figure 2.3. PDMS microfluidic device on a glass coverslip with channel architecture and inlet holes clearly visible.

Section Two: Device Performance and Optimization

As mentioned above, this thesis work began at a time when no demonstration of

*M. smegmatis* cell trapping had been reported in the literature; bacterial single-cell studies had focused on *E. coli* [72] or *B. subtilis* [40], but *M. smegmatis* has distinct morphological characteristics that require optimization of the microfluidic cell trapping
device. Chief among these differences are the slow doubling time of *M. smegmatis* and the waxy cell wall of this organism. Accordingly, cell trapping and media delivery protocols were continually updated and optimized over a period of ~1 year to arrive at a protocol that worked reliably. Additionally, the device architecture itself was eventually changed drastically from the design used in initial experiments. This optimization process is described here, as well as the protocols for performing the live-cell time-lapse microscopy experiments. Other groups have now also begun to perform live-cell imaging studies in *M. smegmatis*, further validating the power of this technique [73].

*Subsection One: Cell Loading and Trapping*

The initial device architecture used in this work was based on the Hasty et al. trapping device for *E. coli* described above [59]. The architecture of this device consists of a 3.0-micron high channel for media delivery, and a looping ‘trap’ region that is adjacent to the main media delivery channel and has a height of 0.9 microns, approximately equal to the diameter of a single *M. smegmatis* cell. A schematic of this device is shown in Figure 2.4 below (adapted from [59]). This device (and all other devices described here) was designed in AutoCAD and fabricated in PDMS from an SU-8 mold as described above. In the earliest cell loading experiments, wild type *M. smegmatis* cells were loaded directly into this device, with no pre-wetting or “priming” of the microfluidic channels with media and with no pre-treatment of the cells other than filling a syringe with a culture of cells in mid-log phase growth (OD 0.5).
During cell loading, 7H9 media containing mid-log-phase cell culture was flowed via syringe, which was either depressed manually or connected to a programmable syringe pump (SyringePump.com) flowing at 20 μL/hour. In this phase, most cells flowed through the device without entering the trap region, but a few cells (<1%) would enter the trap channel and remain immobilized there. After cell loading, the syringe was removed, and 7H9 media was flowed from the ‘media inlet’ at a slower rate of 5-10 μL/hour so that fresh nutrient media would flow into the trap channel while not dislodging cells trapped there. These experiments were performed at 37°C and a cell doubling time of 3-4 hours was observed. Media switching was accomplished by manually switching between syringes feeding into the media inlet; experiments with Rhodamine fluorescent dye revealed that switching operations operated on a timescale of <1 minute (Figure 2.5).
Figure 2.5 Rhodamine dye was flowed into the cell trapping region for 60 seconds and replaced with 7H9 media.

As seen in Figure 2.6 below, this early device was functional in that we were able to successfully trap live *M. smegmatis* cells in the trapping channel using this device and cell loading protocol. However, the slow growth of *M. smegmatis* combined with the waxy cell wall of this organism necessitated changes in the device itself and with the loading/trapping protocol. Although the waxy coating of *M. smegmatis* allows for good adherence to PDMS channels, it also results in cells having a high affinity for each other: in other words, cells have a tendency to flocculate, or ‘clump,’ both when growing in liquid flask culture and when introduced into a microfluidic device [74]. This posed a problem for single-cell imaging, because the fluorescent signal from two clumped cells cannot be easily resolved and quantified. Another consideration apparent in Figure 2.6 is that the cells are sparsely distributed in the trapping channel. This is due to the device architecture: because the cells are not forced through the trapping channel, the vast majority will never enter this channel, and only a few cells will become trapped. With
fast-growing *E. coli*, a sparsely populated microfluidic channel will grow into a dense monolayer within a few hours, so a low initial number of trapped cells is acceptable. However, since *M. smegmatis* doubles every 3-5 hours, a relatively dense monolayer is required to get a meaningful population size for experiments.

Figure 2.6 *M. smegmatis* cells in the trapping channel of the microfluidic device. This trap contains approximately 15 cells, whereas >30 cells are preferred for imaging experiments.

To mitigate these issues, we developed a protocol for centrifuging and filtering cells prior to loading that ensured a high-density population of non-clumped cells for trapping. Cells were grown to log-linear phase growth overnight in a flask; then, 5mL of culture were centrifuged at 3500 rpm for 5 minutes, and the pellet was re-suspended in 1.5 mL of fresh 0.2-micron filter-sterilized 7H9 cell media. This created a dense culture volume, which was forced through a 5um syringe filter (Millipore) to break up any cell clumps. The OD after filtering diminished significantly, to 0.1, as most of the cells were trapped in the 5.0-micron filter.

Although the Hasty-based device functioned well as a cell trap, persistent issues existed with regards to maintaining growth conditions for long time periods in the device.
Because PDMS is a compliant material, hydrostatic pressure dissipates along the length of a PDMS microchannel. The pressures required to do this resulted in high enough flow rates at the cell trapping region of the device that it was difficult to maintain an immobilized monolayer of cells in the trapping region. This problem was compounded by the waxy, adherent cell coating of *M. smegmatis*, which led to a large number of cells adhering to channel walls throughout the device, not just in the trap region. Finally, the sub-micron channel heights required to create a true ‘monolayer’ of cells resulted in PDMS channels that were prone to collapse, because the channels had a width:height ratio exceeding the maximum 15:1 aspect ratio reported for PDMS channels [67].

In order to mitigate these issues we designed and fabricated a device loosely based on a commercially available microfluidic cell trap (CellAsic Corp.) in which the trapping channel is a component of, rather than an adjacent offshoot from, the main flow channel (Figure 2.7), which forces all cells through the trapping region of the device and results in faster and more efficient cell trapping. This trap region is 0.8 microns in height, which allows for some degree expansion of the PDMS channel due to hydrostatic pressure from fluid flow and thus maintaining a true ‘monolayer’ of immobilized cells. The increased trapping efficiency of this device rendered the cell centrifugation step unnecessary, and the centrifugation step was removed from the cell loading protocol. To prevent collapse of the 0.8 micron height trapping channels, support posts were designed into the device to maintain an aspect ratio of 10:1 in the trapping channels. Also, the length of the channels was shortened from 2cm to 0.8cm, to minimize pressure dissipation along the channel length.
Figure 2.7 shows a diagram of the microfluidic device architecture that was used for all experiments from 2011 onward. The dimensions of the channels in the trapping region are 1200 microns (1.2mm) wide, 2000 microns (2mm) long, and 0.8μm tall, with support posts spaced every 15 um to prevent channel collapse. Outside of the trapping region, the device is 3.5μm tall, which helps avoid clogging of channels, and the width and length of these channel segments are 1.5mm and 4mm respectively. Support posts are placed throughout these channels as well, but are not shown in Figure 2.7 in the interest of visual clarity. Media delivery is accomplished via syringes that are actuated by a programmable syringe pump and set to 5 μL/hour flow rates, and media switching can be accomplished by alternative between syringes containing different media formulations as described above. Due to these changes, the new device architecture is robust and offers easier cell trapping as well as live-cell culture capabilities. The data shown in Chapters 3 and 4 was obtained using this device architecture unless otherwise noted.

![Figure 2.7 The CellAsic-style cell trapping device resulted in improved cell trapping efficiency.](image)
Subsection Two: Live-Cell Imaging

The experimental protocol for acquiring time-lapse images was performed as described below, following standard procedures for time-lapse fluorescence microscopy [53, 75]. Deviations from this general protocol were rare, and for the data presented in this dissertation the time-lapse protocol followed this method unless otherwise noted. After cells were loaded into the microfluidic device, they were allowed to grow for 1 hour without any fluorescence image acquisition. This step allowed the cells time to "acclimate," i.e. to recover from any stresses related to filtering/loading, before exposing them to high-intensity fluorescent light, which can be phototoxic to live cells [75]. In our experiments it was observed that cell growth was slowed when fluorescent acquisition was begun immediately after cell loading, especially at short excitation wavelengths, e.g. 440nm for imaging of cyan fluorescence protein (data not shown). Phase contrast images were taken every 5-15 minutes over this time period to ensure that cell growth was visible and to monitor the on-chip conditions before continuing the experiment.

After the one-hour incubation following cell loading, brightfield images were acquired every 15 minutes and fluorescence images were acquired every 30 minutes. When automated image acquisition and shuttering was available (i.e. in all experiments performed from 2011 onward), these images were acquired automatically using the timelapse imaging functionality ("Acquire 6D") included with the Nikon NIS-Elements imaging software. Exposure time varied between experiments depending on the microscope and fluorescence channels used, but was kept consistent between related experiments (i.e. experiments measuring the same strain or phenomenon). In this
dissertation, the acquisition times are reported for each experimental dataset. As described above, device temperatures were maintained at 37 degrees Celsius for the duration of all experiments; this temperature was verified using a thermocouple and each microscopy system was calibrated individually.

Figure 2.8 Screenshot of the NIS-Elements user environment during a typical timelapse experiment.

A screenshot of the NIS-Elements imaging software during a typical experiment is shown above in Figure 2.8. A dialog box allows the user to control camera settings such as exposure time, binning, and visual gain. The “ND Acquisition” dialog box allows
for automated experiments to be set up, with control over fluorescence channel, X-Y multipoint imaging, autofocus, acquisition interval, and Z-stack images. If macros such as autofocus algorithms or simple “wait” steps are desired, they can be programmed and integrated into the ND Acquisition functionality as well. Finally, a live view of the stage (or the last image acquired) is visible, and the brightness/contrast settings for brightfield and fluorescence channels can be manipulated individually. Thus each dataset results in a multi-channel timelapse image file (.nd2 format) that can be subsequently analyzed in the image analysis software ImageJ and/or other image analysis functions in MATLAB, as described in Section Three.

Section Three: Image Segmentation and Analysis

As described in Section Two, data from each time-lapse microscopy experiment was obtained in the form of a multi-channel fluorescence image file in .nd2 format, containing raw image data for each fluorescence acquisition channel at each timepoint. This section describes the MATLAB and ImageJ-based tools that were developed and used for this work [76, 77]. These tools include MATLAB functions developed in the Galagan lab by Wen-Han Yu and Jason Keller, as well as the open-source software tools Miji and MicrobeTracker, which were developed by other groups [78, 79]. Together, these tools constitute a modular and automated pipeline for extracting and visualizing quantitative information about single-cell morphology, growth, division, lineage, and fluorescent protein expression from the .nd2 files obtained in these time-lapse imaging experiments. See Appendix for all code described in this Section.
In order to prepare raw .nd2 files for image segmentation in MicrobeTracker, basic processing steps including image registration (i.e. alignment) and background subtraction must be performed. This can be accomplished via the MATLAB program ProcessImage_byJ.m, originally written by Wen-Han Yu and modified by Jason Keller. This program takes as input a raw .nd2 file and parameters indicating which fluorescence channels were acquired during the timelapse experiment. The program proceeds in four steps, using the open-source software Miji to interface directly with ImageJ at each step. Step 1 opens the .nd2 file and organizes each fluorescence channel into a separate folder, with each timelapse image frame saved as a separate .tif. For example, for an .nd2 file with 60 frames in brightfield and in GFP fluorescence, Step 1 will create folders called ‘BF’ and ‘GFP’, each containing 60 .tif images. Step 2 uses the ImageJ function StackReg to align the frames of each image stack, which corrects for frame-to-frame jitter and image drift and is critical for subsequent cell segmentation. In Step 3, a background subtraction operation is performed on all images to further aid in cell segmentation by increasing contrast. Finally, Step 4 creates a single .tif file for each image stack, e.g. one file called ‘BF.tif’ containing all 60 processed brightfield frames and one file called ‘GFP.tif’ containing 60 processed GFP image frames. These multiframe .tif files can be directly read by MicrobeTracker in the downstream processing steps. Thus, the function ProcessImage_byJ.m is a simple tool to automate several image processing steps that could otherwise be performed manually in ImageJ, but at the possible expense of uniformity, accuracy, and speed.
**Subsection Two: MicrobeTracker**

MicrobeTracker is an open source software tool developed at Yale University for the purpose of segmenting images of bacterial cells [79]. It is designed to outline and track rod-shaped bacilli over the course of a time-lapse image series, and to store quantitative information about the morphology, growth, division, lineage, and fluorescence intensity of each individual cell. The software runs in MATLAB and can be modified for optimal performance based on the shape parameters of the cells and other characteristics of the images (e.g. brightness, contrast, and magnification).

A screenshot of the MicrobeTracker program in use is shown below in Figure 2.9. A frame of a brightfield image series is displayed the main window of the program, with a monolayer of rod-shaped cells as well as circular PDMS support posts visible. The cells have been automatically traced by MicrobeTracker, and these traces appear as white outlines. Each cell is numbered, and the information panel at the bottom-left indicates that 104 cells were traced in total across the 53 image frames. Also displayed in this information panel is information regarding the current frame (19 of 53), the currently selected cell (Cell 23 of 104), and some information about the currently selected cell. Using this interface, the user can automatically trace all cells in each image frame and manually correct any cells not correctly traced or identified by the program. The program saves all information to a .mat file that stores all the data for each cell analyzed. This file can then be processed in MATLAB to generate single-cell fluorescence plots and all other graphs, as described in Subsection Three.
Figure 2.9. Screenshot of MicrobeTracker program. Cell 23 is marked by the black arrow.

Subsection Three: MATLAB Processing/Analysis

Once the .mat file containing all cell information for a given timelapse experiment is generated in MicrobeTracker, the data must now be extracted for further processing by subsequent MATLAB programs. The program getCellTrace.m was written by Jason Keller for this purpose. getCellTrace.m employs a switch-case loop to store information not encoded by MicrobeTracker, such as the fluorescence acquisition time interval, experimental conditions (e.g. antibiotic concentration), and the locally-stored data path of the results file. The program can thus be called using an experimental ID number, e.g. '02-04-14', which matches the '02-04-14' case in the switch-case loop. The output of this program is two cell arrays, "CellFluore" and "ExptData," which are leaner data structures
than the results file and which encode all relevant cellular and experimental information. The program getCellTrace.m can therefore be thought of as a data-cleaning step that can be modified based on the specific needs of the researcher while keeping the MicrobeTracker output file intact.

A group of modular analysis/plotting functions were written by Wen-Han Yu and Jason Keller to perform specific operations on the single-cell data structures and generate the graphs presented in this dissertation. As a representative example, the function DrawGrowthRate.m creates two figures based on the data in CellFluore and ExptData: the first figure plots the length of each individual cell over time, and the second figure plots the average growth rate of the population as a function of time. The user can specify only a subset of cells to be analyzed, e.g. for comparing the growth behavior of cells that survived drug treatment vs. cells that lysed or died. The graphs generated by this program for a representative experiment are displayed below in Figure 2.10. Plot information such as the title, number of cells plotted, and drug treatment time is automatically generated from the information contained in ExptData, allowing for streamlined and uniform processing of each experimental dataset.
Additional MATLAB functions were written to generate plots of information such as single-cell fluorescence intensity, the number of fluorescence ‘peaks’ for each cell during an experiment, and live/dead statistics for each experiment. These functions are not described in detail here, as they operate similarly to DrawGrowthRate.m, but the figures in Chapters 3 and 4 were created using these functions. Together, the computational tools described in this Section form an automated image processing pipeline that is simple to use, fast, accurate, and modular, in the sense that it can be easily adapted for new functionalities as needed simply by writing additional plotting or analysis scripts. Indeed, several such scripts were created over the course of this thesis work (e.g. the fluorescent signal peak-counter function described above), and the time required to create a new plotting function was approximately 4-8 hours on average.
Section Four: Molecular Cloning in *M. smegmatis*

Demonstrating the ability to trap and culture live *M. smegmatis* cells in a microfluidic device for >24 hours was an essential milestone for studying cellular behavior of *M. smegmatis* in real-time. Concurrently, molecular cloning work in *M. smegmatis* was performed in order to generate engineered strains containing genetically encoded fluorescent protein (FP) reporter molecules for genes of interest. This Section summarizes the methods used to generate strains of *M. smegmatis* for the experiments described in this dissertation.

**Subsection One: Description of Protocols**

In general, the protocols described here follow standard molecular cloning practices and references [80, 81]. Cloning protocols designed specifically for Mycobacteria are modified slightly versus those used with *E. coli* due largely to the reduced permeability of the mycobacterial cell membrane and the slow growth of Mycobacteria species; for the methods described herein that are specific to Mycobacteria, protocols were followed according to the handbook *Mycobacteria Protocols* [82].

Primers were designed using genomic sequence data taken from TBDB (tbdb.org) or NCBI databases (http://blast.ncbi.nlm.nih.gov/). All primers were ordered from Integrated DNA technologies (http://idt-dna.com/) with appropriate restriction enzymes chosen based on the plasmid backbone; this analysis was performed using ApE (http://biologylabs.utah.edu/jorgensen/wayne/apec). Primers were used to amplify genomic DNA using standard polymerase chain reaction (PCR) methods [81]. PCR
products were visualized on a 0.1% agarose gel and the correct size band was excised and purified using the Qiagen gel extraction kit, and all DNA digestions and ligations were performed using standard Qiagen kits and protocols (http://www.qiagen.com/). Ligation products were again PCR amplified for increased concentration and purity before being digested and ligated into vector backbones. Vectors containing the plasmid insert were transformed into the competent *E. coli* Top 10 strain using standard heat-shock transformation protocols (42°C for 60-75 seconds followed by 1 hour of recovery at 37°C) and cells were plated and screened using antibiotic-LB agar plates.

Plasmid DNA from screened *E. coli* colonies was collected using Qiagen kits and procedures (www.qiagen.com/) and verified by sequencing (www.macrogenusa.com/) before being transformed into electrocompetent *M. smegmatis* using standard electroporation protocols as described in [82]. *M. smegmatis* transformants were screened on antibiotic-LB or antibiotic-7H9 agar plates. For strains containing fluorescent protein constructs, fluorescence was verified via fluorescent emission measurements using a Tecan Infinite 200 microplate reader (http://www.tecan.com/) and/or a fluorescent microscope with the appropriate filter settings (Nikon).

*Subsection Two: Inducible Promoter Systems and Fluorescent Protein Constructs*

There now exist numerous systems of exogenous control of gene transcription in living cells, in prokaryotes as well as eukaryotes [83]. These are known as inducible gene expression systems because they allow for reversible control of gene expression via the addition of an inducer chemical. The Tet, or tetracycline-controlled, expression system
(Clontech) is an inducible promoter system that is responsive to the antibiotic tetracycline and its derivatives (e.g. doxycycline) [84]. In this system, a promoter containing tetracycline-responsive elements is cloned upstream of the gene of interest. Also cloned into the cell is a vector that constitutively expresses the TetR protein, which can be an activator (Tet-On) or a repressor (Tet-Off). When anhydrous tetracycline (aTc) is introduced into the cell culture medium, the aTc molecules bind to the Tet protein, causing a conformational change that allows it to bind to the Tet-responsive elements in the gene of interest’s promoter. In the case of the Tet-On system, this complex then recruits RNA polymerase to the promoter, “turning on” transcription of the downstream gene. These systems have been shown to enable exogenous control of gene regulation over two orders of magnitude in Mycobacterium smegmatis as well as in MTB [84]. A further advantage of the Tet system is low basal transcriptional levels in un-induced cells (i.e. low ‘leakiness’ of the system).

We employed Tet-inducible systems to facilitate the study of transcription factor activity in M. smegmatis at the single-cell level in a microfluidic platform. Using the molecular cloning procedures outlined in Subsection One, our group designed and constructed strains of M. smegmatis where the TF KstR is under transcriptional control of the Tet-On system, such that the TetR protein binds to the TetO promoter and inhibits transcription of KstR except when aTc is present (i.e., addition of aTc ‘turns on’) KstR. These strains were designed and constructed by Suma Jaini. We also cloned green fluorescent protein (GFP) into the vector downstream of KstR, so that we can measure GFP production as an analogue for KstR’s production. A hygromycin resistance vector
ensures the ability to maintain this vector in cloned cell populations.

**ATC Addition Activates Tet-KstR-GFP Transcription**

(Plate Reader Measurements)

![Graph showing RNA levels over time with ATC addition](image)

**ATC Addition Activates Tet-KstR-GFP Transcription**

(Microfluidics Measurements)

![Graph showing fluorescence levels over time with ATC addition](image)

**Single Cell Images**

![Single cell images at different time points](image)

Figure 2.11. Representative plate-reader and microfluidics experiments showing repeated transcriptional activation of Tet-KstR-GFP in response to ATC addition (50 ng/mL).
We verified the performance of the Tet-driven KstR-GFP system using plate reader measurements (Tecan) and in single cells using our microfluidic live-cell imaging platform, as shown above in Figure 2.11. In both the plate reader and microfluidics experiments, addition of aTc causes a 4-fold increase in relative GFP fluorescence within 2 hours of ATC addition. In the absence of aTc, KstR production (and thus, GFP fluorescence) is negligible, and a Clp proteasome degradation tag targets the KstR-GFP for degradation [85, 86]. In the microfluidics experiment, media switching was performed by manually switching between syringes containing 7H9 media with and without ATC (50 ng/mL in the experiment shown in Figure 2.11) as described above. This was performed on the fly during experiments and did not dislodge cells from the trap region. These initial experiments served to validate the performance of our inducible promoter and fluorescent protein constructs, and formed the basis of the strains that were engineered for the experiments described in Chapters Three and Four.

Subsection Three: List of Strains

A list of the strains and plasmids used in this work is given below in Table 1. The E. coli strain used for all cloning was the Top 10 strain (Invitrogen). M. smegmatis clones were constructed from a wild-type mc² 155 strain [87]. Plasmids were based on the episomal plasmid PMV261 or the integrating plasmid PMV306, and the strain used for each experiment in the text is indicated [88].

It is important to note that because the episomal plasmid PMV261 is not integrated into the chromosome, it may be present at a different copy number in different
cells. This precludes quantitative comparison of cell-to-cell heterogeneity in gene expression. Therefore, the integrating plasmid PMV306 was used in this work except where noted. This plasmid integrates at the chromosomal att site using an int phage integrase, and is thus present at a copy number of exactly 1 in every cell. For example, Figure 2.12 below shows a schematic of the HspXp:ECFP reporter construct in the integrating PMV306 plasmid with hygromycin resistance. Additionally displayed in this schematic is the PstI restriction site used during cloning, the E. coli origin of replication OriE, and the int sequence for integration into the M. smegmatis genome.

Figure 2.12. Schematic of a PMV306 integrating vector used in this work.
Table 1. Mycobacterial strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
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<td>[87]</td>
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<td>ΔKstR::Zeocin(^f), pKG1, p6038-2</td>
<td>This study</td>
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<td>Invitrogen</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMV306H</td>
<td>Hyg(^f)</td>
<td>[88]</td>
</tr>
<tr>
<td>pMV261K</td>
<td>Kan(^f)</td>
<td>[88]</td>
</tr>
<tr>
<td>pWOscKC1953</td>
<td>pMV261K derivative, (P_{nit/tet-NitR-m}), (P_{nit/tet-TetR-f}), (P_{nit/tet-KstR-RFP-m}), (P_{M1953-YFP-s})</td>
<td>Yu et al. (manuscript in preparation)</td>
</tr>
<tr>
<td>pWOscKC6038</td>
<td>pMV261K derivative, (P_{nit/tet-NitR-m}), (P_{nit/tet-TetR-f}), (P_{nit/tet-KstR-RFP-m}), (P_{M6038-YFP-s})</td>
<td>Yu et al. (manuscript in preparation)</td>
</tr>
<tr>
<td>JK306H</td>
<td>pMV306H derivative, (P_{MHispX-ECFP})</td>
<td>This study</td>
</tr>
<tr>
<td>pKG1</td>
<td>pMV306H derivative, (P_{ter-KstR-RFP-m})</td>
<td>This study</td>
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<tr>
<td>p6038-2</td>
<td>pMV261K derivative, (P_{M6038-GFP-s})</td>
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</tr>
<tr>
<td>pNar:ECFP</td>
<td>pMV261K derivative</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^f\): fast proteasome degradation tag; \(^m\): medium degradation tag; \(^s\): slow degradation tag.

PM1953: Intergenic region upstream of MSMEG 1953.
PM6038: Intergenic region upstream of MSMEG 6038.
PMHspX: Intergenic region upstream of MSMEG 3932.
pNar: Intergenic region upstream of *E. coli* gene narG (ECK1218).
CHAPTER THREE: Quantitative Time-Lapse Imaging of *M. smegmatis* Cells

As described in Chapter 1, the main advantages of a single-cell time-lapse imaging platform include the ability to extract quantitative data about diverse cellular parameters (e.g. gene expression, growth, morphology, lineage, cell fate), simultaneously and with high throughput, and to use this data to more accurately model or understand dynamic processes within single cells and to observe to what extent heterogeneity in these parameters exists within a population of cells. After individually validating the separate components of our experimental platform – the microfluidic cell trap itself, the fluorescent *M. smegmatis* strains, the live-cell microscopy system, and the automated image analysis pipeline – we used the platform to address several questions of this nature, the results of which are discussed here and in Chapter 4.

Using our platform, we analyzed in real-time the functional relationship between the transcription factor KstR and two of its computationally predicted gene targets, resulting in what is, to our knowledge, the first direct measurements of TF-target interactions in single *M. smegmatis* cells. This work employed a novel synthetic oscillator construct developed by Wen-Han Yu to endogenously modulate KstR transcription; this oscillator has tunable pulse amplitude and frequency, based on the concentration of inducer chemicals (manuscript in preparation). We additionally analyzed cell growth, morphology, and cell division/lineage data for 800 *M. smegmatis* cells to determine the relationship between cell growth and division timing. This work lends insight to a current debate about whether *M. smegmatis* cell division is governed primarily by time-based or size-based control mechanisms [49, 51]. Our results are a compelling demonstration of
the diverse types of information that can be obtained using quantitative single-cell measurements, specifically with respect to the cell biology of Mycobacteria.

**Section One: Quantifying KstR's Regulation of Predicted Target Genes**

As described in Chapter 1, the Galagan group used ChIP-Seq based techniques to reconstruct the first genome-scale map of the M. tuberculosis gene regulatory network, identifying global binding sites for >100 transcription factors [24]. This work resulted in thousands of predicted regulatory interactions and led to several key insights about links between biological pathways in Mycobacteria. However, ChIP-seq alone provides information only about associations between TFs and putative target genes, not about the nature of gene regulation (activation or repression) [89]. In effect, ChIP-seq mapping provides the topology/architecture of the regulatory network, but little information about the functional annotations of network edges. To gain further insight about the functional relationship between regulators and their gene targets – of critical importance for using the network map to make predictions about biology – requires integrating the topological data provided by ChIP-Seq with complementary systems identification techniques. The experiments described here are a demonstration of this type of integration, and our results enabled direct validation of gene regulatory interactions in single Mycobacteria cells.

**Subsection One: Tet-Inducible KstR**

The TF KstR has been shown to control a regulon that is involved in lipid metabolism pathways, especially cholesterol degradation, in *M. tuberculosis* and in *M.*
smegmatis [90]. KstR is part of the extended hypoxic response in MTB [91], and MTB is known to utilize host-derived fatty acids in vivo [92]. ChIP-Seq analysis revealed direct connections between the hypoxic response and lipid metabolism, mediated by the TFs Rv0081 and Rv0324 [24]. For these reasons, our group was interested in exploring the regulatory interactions of KstR in order to further elucidate its role in MTB pathogenesis.

To perform these studies, we analyzed genetically engineered strains of *M. smegmatis* using the microfluidics system described in Chapter 2. We first used strains of *M. smegmatis* containing a Tet-inducible KstR-GFP fusion protein in a KstR mutant background; these strains additionally incorporated a construct containing the promoter for the gene MS6038 fused to the gene for red fluorescent protein (RFP) in the episomal plasmid PmV261. These strains were designed and built by Suma Jaini. KstR was predicted via ChIP-Seq and overexpression microarray experiments to repress expression of the gene MS6038. Consistent with this prediction, induction of KstR-GFP in single *M. smegmatis* cells led to repression of the MS6038 promoter and a concomitant decrease in RFP expression. This experiment was repeated at varying ATC concentrations (10, 50, and 100 µg/mL) and with *M. smegmatis* strains containing proteasome degradation tags of varying strength attached to the FP constructs as described in Chapter 2. A representative dataset from these experiments is shown in Figure 3.1, where the strain is KG-1/6038-2 and the ATC concentration is 50 ng/mL. Because the PMV261 plasmid is present at different copy numbers in different cells, direct cell-to-cell comparison is not possible in these data; however, the repressive effect of KstR on 6038_RFP transcription was observed in >90% of the cells studied across all experiments. These experiments
served as proof of concept for the microfluidics platform itself, suggesting that it was indeed possible to observe gene regulation taking place within individual *Mycobacteria* cells in real time using this experimental setup.

**Figure 3.1.** Movie montages showing expression of Tet-inducible KstR_GFP and MS6038p_RFP after addition of ATC. As KstR is produced, expression of the KstR target MS6038 is repressed.

*Subsection Two: Oscillator-Driven KstR*

To further explore the relationship between KstR and the gene MS6038, we next used a construct containing a synthetic 'gene oscillator' architecture driving expression of KstR (designed and built by Wen-Han Yu; manuscript in preparation). In the gene oscillator construct, linked positive and negative feedback loops provide robust and tunable oscillations in the transcriptional activity of KstR. The motivation for using a gene oscillator was to gain the ability to provide endogenously-controlled periodic inputs to the system, thereby making it possible to observe frequency response characteristics of the system while being less sensitive to noise [93-95]. By modulating KstR concentration
at varying frequencies and measuring the dynamic responses of its regulated downstream elements, the frequency response characteristics of these elements can be measured. Though oscillatory gene expression systems had previously been built in *E. coli* [59, 96, 97] and in mammalian cells [98], this work was the first such effort in Mycobacteria.

![Figure 3.2. Schematic showing architecture of the synthetic oscillator circuit.](image)

The oscillator circuit (Figure 3.2) consists of linked positive and negative feedback loops that utilize the repressor TetR, which represses transcription of the *TetO* promoter and is responsive to the chemical aTe as described in Chapter 2, and the activator NitR, which activates transcription of the *nitA* promoter and is responsive to the
chemical Caprolactam (Cap) [99]. A chimeric promoter region \( (P_{nitr/te}) \) was constructed that contains an activation operator site from the \( nitA \) promoter and two repression operator sites from the \( tetO \) promoter. The binding affinity of both NitR and TetR are modulated by the concentration of their inducer chemical (Cap and aTc, respectively), where Cap increases the binding affinity of NitR for the \( nitA \) promoter and aTc decreases the affinity of TetR to the \( tetO \) promoter (and therefore, addition of aTc de-represses \( TetO \) activity) [84]. This chimeric promoter therefore acts in a competitive manner whereby the activator NitR and the repressor TetR compete for binding to the promoter. Critically, the dimerization of TetR necessary for its activity introduces an extra delay in the repressor component of the gene circuit, giving rise to the oscillatory behavior. Oscillatory behavior of this circuit was predicted computationally and verified in single-cell measurements using the microfluidics system (Figures 3.3, 3.4).

![Graph](image)

**Figure 3.3.** Computational prediction of gene expression from the oscillator circuit, and experimentally obtained single-cell fluorescent traces from cells containing the circuit. Inducer concentrations: 25 ng/ml aTc, 14 mM Cap.
Figure 3.4. Representative image series of oscillatory KstR-RFP expression in single cells.

At constant inducer concentration, single cells were found to exhibit sustained oscillations of KstR-RFP as predicted in the computational simulations. In total, 18 aTe/Cap concentrations were studied. In each experiment, ~70% of individual cells on average displayed robust oscillations over 24 hours of measurement with an average period of 4.5 hours (Figure 3.4). Cells that did not exhibit oscillations either exhibited monotonically decaying fluorescence, or undetectable fluorescence. Heterogeneous cellular responses in an isogenic cellular population have been widely observed and can be attributed to such cell-to-cell differences in cell size, age, cell cycle stage, and growth.
rate; unequal partitioning of cellular components in dividing daughter cells; and extrinsic and intrinsic sources of noise in gene expression [38, 49, 100]. Variations in oscillatory period, timing and amplitude across the population can also be attributed to these factors. In addition, the plasmid used in these studies was based on the episomal pmV261 background and plasmid copy number thus varied from cell to cell, which provides another source of cell-to-cell variation in the population. Previous work in *E. coli* reduced this variation by increasing the plasmid copy number to ~50 copies/cell, which resulted in robust oscillations in >99% of cells [59]. Raising the number of gene copies per cell was also shown decrease gene expression noise in *Bacillus subtilis* [101]. Further work could be done in *Mycobacteria* to increase plasmid copy number in this manner, or alternatively the oscillator circuit could be placed in an integrating plasmid (e.g. pMV306) to ensure that each cell contains exactly one copy of the circuit components, although this could still be subject to intrinsic noise due to “bursty” mRNA production, especially since expression in this circuit is controlled via the balance of competing positive and negative regulatory elements [102].

After the functionality of the synthetic oscillator circuit was demonstrated using KstR-RFP, new strains of *M. smegmatis* were constructed to explore the temporal dynamics of KstR regulatory interactions with two of its predicted target genes. For this purpose, two genes (MS1953 and MS6038) were selected; these were predicted by ChIP-Seq to be directly regulated by KstR [24]. MS6038 is a predicted hydroxylase related to the MTB oxidoreductase gene Rv3570c, and MS1953 is a WhiB-like transcriptional regulator and is involved in cholesterol catabolism (annotations taken from tbdb.org).
The upstream promoter regions of MS1953 and MS6038 were transcriptionally fused with a yellow fluorescence protein (YFP) gene as described in Chapter Two. All synthetic circuit components were built into the same plasmid backbone to maintain identical copy numbers of the components in individual cells.

Using our microfluidics platform, we performed time-lapse fluorescent microscopy experiments on these strains (abbreviated here as KstR-1953 and KstR-6038) and obtained multi-color fluorescent images for >24 hours at single cell resolution. Representative single-cell traces of RFP and YFP fluorescence from one experiment (KstR-1953) are shown below in Figure 3.5. Each experiment resulted in single-cell data for ~50 cells, and over the course of all experiments we obtained 798 single-cell traces (376 single-cell traces for KstR-1953 and 422 single-cell traces for KstR-6038). These data traces contained information about both the “input” (i.e. KstR-RFP) and the “output” (the gene target, i.e. either MS6038-YFP or MS1953-YFP) and were processed and analyzed using the computational pipeline described in Chapter 2.

Figure 3.5. Representative single-cell traces of KstR-RFP and d(MS1953-YFP)/dt.
The responses of both MS-1953 and MS-6038 showed distinct dynamic behaviors corresponding to the KstR input. We measured this quantitatively by computing the cross correlation between KstR and its gene target; the cross-correlation is a measure of similarity of two waveforms as a function of a time-lag applied to one of them, and can therefore be used to visualize the relationship between the two fluorescence signals obtained in our single-cell data traces [72]. It was found that KstR-MSMEG1953 showed a positive correlation with a lag time of 1 hour, whereas KstR-MSMEG6038 displayed a negative correlation with a lag time of 1.25 hours (Figure 3.6).

![Figure 3.6. Cross-correlations show regulatory activity of KstR on two of its targets.](image)

Interestingly, microarray data in a KstR knockout [90] had identified MS6038 as a strong repression target (MS6038 expression increased 136-fold in the KstR knockout) but did not indicate that MS1953 was a member of the KstR regulon, and KstR ChIP-Seq binding data for the two genes also suggested much stronger binding of KstR to 6038.
than to MS1953 (relative ChIP peak heights of 92% and 1.3% respectively) [24]; and yet, comparison of the cross-correlation functions (1h vs. 1.25h) suggests that KstR directly regulates MSMEG1953, instead of controlling it via some secondary regulatory pathway within the network. This result demonstrates that even relatively weak TF-target binding, as evidenced by low ChIP peaks, can indicate direct regulation. Additionally, KstR’s role as an activator of 1953 is an important result because KstR has been shown to generally function as a repressor; this work thus shows bi-functional activity (activation and repression) for a single TF. In the case of MS1953, its regulation by KstR is reasonable given MS1953’s role in cholesterol catabolism and KstR’s known role as a regulator of cholesterol metabolic pathways. Further exploration of the KstR regulon (and indeed the regulons of other key mycobacterial TFs) may yield additional surprising insights.

**Section Two: Morphology and Growth Measurements in Single Cells**

A major advantage of the microfluidic single-cell analysis platform is the wealth of data that can be obtained in a single experiment, not only in terms of throughput (i.e. measuring dozens of cells simultaneously) but in terms of the diverse types of data that are acquired. In particular, information about cellular morphology, growth, division, and lineage can be obtained at the same time as time-lapse fluorescence measurements. These measurements can be used to better understand *M. smegmatis* biology, separately from insights about gene regulation that are obtained. Thus, in the oscillator work described above, we obtained morphology, growth, division, and lineage data for 800 cells, and this data was analyzed via the MATLAB image processing pipeline.
In this analysis, our data was compared to recent reports of single-cell measurements in *M. smegmatis* [49, 51, 103]. In particular, two recent reports – one written by the McKinney group at the Swiss Federal Institute of Technology, Lausanne, and one from the Fortune group at Harvard School of Public Health – showed conflicting results with respect to the organization or ‘rules’ governing cell growth and division in *Mycobacteria* [49, 51]. Fortune et al. reported observations that suggested cell division in *M. smegmatis* is regulated not by size-based control, as in *E. coli*, but rather by a time-based control mechanism [49]. In other words, rather than cells needing to reach a certain minimum size before the cell division process began, *M. smegmatis* cells in their experiments displayed time-based synchronized division in single cells that was not correlated with cell size. In contrast to this report, McKinney et al. observed three things that suggested division in single *M. smegmatis* cells was indeed related to cell size – namely, that birth size and division time were negatively correlated, and that faster-growing cells divided more quickly [51]. These trends more closely reflect the size-based control mechanisms of *E. coli*, because cells that are small at birth must grow more before they reach the requisite size for cell division [104].

With data from our microfluidics experiments, we were able to put our single-cell morphology and cell division measurements in the context of this current debate. It is important to note first that we observed canonical features of *M. smegmatis* growth and division that agreed with those reported in the literature and our own flask-culture experiments [82]. Among these observations were a doubling time of 3-4 hours, asymmetric cell size between daughter cells after division events, and the classic “V”-
shape of cells after division, as can be seen in Figure 3.7, which shows images of a growing *M. smegmatis* microcolony in our microfluidic device, with individual cells segmented and numbered by MicrobeTracker. Additionally, because our analysis pipeline enables lineage tracking, we can directly measure differences in morphology, cell growth, and fluorescence between daughter cells (Figure 3.8). This figure shows the lineage stemming from one cell, “Cell 4,” in a microcolony of *M. smegmatis* cells and plots the cell size of each cell in the microcolony over time. Division events are clearly identifiable as a sudden decrease in cell size.

Figure 3.7. Image series of single *M. smegmatis* cells showing cell segmentation and numbering, as well as the characteristic V-shape of newly divided cells.
That our observations of basic single-cell growth and division characteristics agree with previously published reports was reassuring but not unexpected. If anything it served as an additional indicator that our platform did not impose additional stresses on single cells relative to flask culture. More surprising, though, was the degree of heterogeneity among single cells with respect to these measurements (cell size, growth rate, and division timing). For the 800 cells measured in this study, cell size at birth ranged from 1 micron to 4.5 microns, with a mean value of 2.5 microns. Interdivision time ranged from 2-6 hours for the majority of cells, with a mean value of 4 hours, but we also observed slowly dividing cells with a division time of >10 hours. This considerable heterogeneity between single cells agrees with the observations of McKinney and Fortune [49, 51], and as speculated by Fortune et al., could be a factor underlying the differential susceptibility of single cells to antibiotic treatment.
After generating morphology, growth and division data for 800 cells, we explored correlations between cell size at birth, cell size at division, growth rate, and interdivision time in order to compare our results to those previously reported [49, 51]. Our observations are summarized in Figure 3.9. Strikingly, we found a positive correlation between cell size at birth and cell growth rate (a), and a negative correlation between cell size at birth and division time (b). Additionally, we observed that faster-growing cells divide more quickly than slowly-growing cells (c). These observations generally agree with those reported by McKinney et al., namely, that size-based control mechanisms of cell division seem to be in effect in *M. smegmatis* [51]. We also observed that cell size at birth and cell size at division were positively correlated (d), which is not explained by strict size-control mechanisms of division timing. If division were solely a function of reaching a ‘minimum’ cell size, then all cells would divide after reaching that minimum cell size, regardless of what their size had been at birth. Further work is needed to address this question, but as discussed by McKinney et al. in [51], cell division could have a size-based probability of occurrence after some minimum cell size is reached; because larger cells grow faster, this could lead to the positive correlation between cell size and birth and cell size at division that was observed in our data. While further work is needed to fully elucidate the mechanisms underlying size control and cell division in *Mycobacteria*, from this work and from the two cited reports it is clear *M. smegmatis* grows and divides differently than better-studied model organisms such as *E. coli*. 
In summary, the work presented in this Chapter demonstrated that single-cell studies of *M. smegmatis*, enabled by the microfluidics platform we developed, can be used to study diverse phenomena in single cells, and that the insights from single-cell experiments can lead to improved understanding of gene regulation, regulatory network structure and function, and processes of growth and division. Goals for future work using this methodology could include more comprehensively probing the KstR regulon, or to
provide direct validation of TF regulatory network motifs (e.g. feed-forward loops) [33] that were predicted using ChIP-Seq and microarray data. Another extension of this work, analyzing single-cell responses to stress using morphological and time-lapse fluorescence data, is presented in Chapter 4.
CHAPTER FOUR: Behavior of *M. smegmatis* in Response to Diverse Stresses

This chapter reports observations from stress-response experiments performed using a microfluidic single-cell analysis platform. It will be organized into two sections, based on the type of stress to which the cells were exposed. The first section discusses cellular responses to antibiotic stress, namely D-Cycloserine (DCS). We found that individual cells exhibit considerable differences in their responses to D-Cycloserine-induced stress, and that these differences have implications for cellular survival. The second section discusses hypoxia-induced stress responses in *E. coli* as well as in *M. smegmatis*, and also described a modified microfluidic device that was constructed for these experiments that affords the ability to control oxygen tension in the on-chip microenvironment.

Section One: HspX Expression and Cell Fate Under Antibiotic Stress

In MTB, the heat-shock like protein HspX (also called Acr, alpha-crystallin), Rv2031c, is the most strongly-induced gene in the DosR regulon [105], and it is rapidly induced to ~80-fold expression at low oxygen tension (Figure 4.1, adapted from [26]). A 2006 study found increased growth of an HspX deletion mutant *in vivo* and postulated that that hspX plays an active role in slowing the growth of MTB during the transition to non-replicating persistence [106]. The precise function of HspX is unknown, but it exhibits homology to chaperonin proteins that are involved in prevention of protein misfolding under stress conditions; important to note is that HspX is not induced by heat shock [107]. Based on these findings it is thought that HspX could function similarly to
other heat-shock proteins but in response to hypoxia and other host-mediated stresses as opposed to heat. Supporting this idea, HspX was found to be required for MTB growth in macrophages [108].

![Alpha-crystallin induction at low oxygen saturation](image)

**Figure 4.1. Induction of HspX at low oxygen tension.** Adapted from [26].

The strength of the regulatory link between DosR and HspX is such that HspX is commonly used as a surrogate indicator of DosR regulon induction [109, 110]. In MTB, DosR is by far the strongest transcriptional regulator of HspX, though evidence also exists for binding of the HspX promoter by Rv0767c and Rv2745c, albeit with far weaker coverage (Figure 4.2, adapted from tbdb.org). Similarly, HspX was highly upregulated (6-fold) in an inducible DosR microarray experiment whereas similar experiments with inducible Rv0767c and Rv2745c resulted in -0.7 fold change of HspX expression [24]. It is not known if the regulatory architecture upstream of HspX is identical in MTB and in *M. smegmatis*, and additionally it is possible that as-yet uncharacterized TFs will strongly
bind to the HspX promoter. Further work (perhaps using techniques similar to those presented in Chapter Three) will be needed to precisely characterize the transcriptional regulators of HspX in MTB and in *M. smegmatis* and to arrive at a mechanistic understanding of the function of this protein. However, all studies cited above point to the utility of HspX as a reporter of DosR regulon induction.

**Predicted Regulatory Interactions to Rv2031c**

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<th>Product</th>
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<th>Coverage</th>
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<tr>
<td>Rv2745c</td>
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<td>Rv3133c</td>
<td>devR</td>
<td>two component system transcriptional regulator</td>
<td>1.047, 132, 3055.1953</td>
<td>20.8372, 38.9189</td>
<td>downstream, upsteam</td>
</tr>
</tbody>
</table>

Figure 4.2. Predicted transcriptional regulators of *HspX*. Taken from tbdb.org.

To study stress responses of single *M. smegmatis* cells, we constructed an *M. smegmatis* strain containing a promoter-FP construct for measurement of HspX transcriptional activity. This construct was named JK306H and contains a copy of the *M. smegmatis* HspX promoter fused to the sequence for an enhanced cyan fluorescent protein (ECFP) in the integrating plasmid pMV306 with a hygromycin resistance marker. Thus, CFP fluorescence is an indicator of transcriptional activity at the HspX promoter. We were interested in studying the response of *M. smegmatis* to diverse stresses, including hypoxia and other host-induced stresses as discussed in Section Two, as well as antibiotic stress. To this end, we treated a population of log-phage *M. smegmatis* cells with the antibiotic D-cycloserine (DCS) and measured the effect of the drug on cell growth, HspX expression, and cell fate (via live-dead staining). DCS is a second-line
antituberculosis drug that inhibits cell wall biosynthesis via the inhibition of two enzymes [111]. These enzymes are alanine racemase (Alr) and d-alanine d-alanine ligase (Ddl), which are involved in synthesizing peptidoglycans [112]; for instance, Alr converts L-alanine to D-alanine, and it was found that Alr mutants of MTB required supplementation of growth medium with D-alanine for survival [113] and showed survival defects in vivo.

The protocol for drug treatment experiments was as follows. Overnight culture (OD 0.4-0.6) of M. smegmatis cells were loaded into a microfluidic device in antibiotic-free 7H9 medium and allowed to grow in a monolayer at 37 degrees for one hour. After one hour in the device, fluorescence acquisitions at 100X magnification were begun, with 30 minutes between exposures. Simultaneously, brightfield images were acquired every 15 minutes. For the data presented here, t=0 is equivalent to the time at which fluorescent exposures were begun (i.e. the one hour ‘acclimation’ period is not included in the datasets). Cells grew in antibiotic-free medium for 3 hours, at which point the cells were exposed to 7H9 media containing DCS at the indicated concentration for several hours (10-100 ug/mL; the minimum inhibitory concentration of DCS for M. smegmatis is ~40 ug/mL). After drug treatment was sustained for 7-10 hours (as denoted for each experiment), DCS was removed and cells were allowed to recover in antibiotic-free 7H9 medium. At the conclusion of these experiments, propidium iodide (PI) staining was performed. PI is a red fluorescent dye that permeates degraded cell membranes and thus serves as an indicator of cell death [114].

Surprisingly, we observed that addition of DCS caused rapid and strong induction of HspX transcription in some M. smegmatis cells, as observed by an increase
in ECFP fluorescence (Figure 4.3). Fluorescence was observed in high-intensity 'pulses' of ECFP, denoting strong yet transient transcriptional activation of the HspX promoter. These fluorescence pulses occurred in a minority of the cells studied, approximately 30-50% of all cells, and although the majority of pulsing cells exhibited exactly one pulse of fluorescence, 5-10% of pulsing cells exhibited >1 fluorescence pulse. In the experiment depicted in Figure 4.3 below, 30 ug/mL DCS was introduced to the microfluidic device at time t=3.5 hours after fluorescence acquisition began, and was removed at t=13.5 hours. Figure 4.4 shows image frames from this same experiment, with ECFP pulses in response to DCS addition visible in several cells.

**Figure 4.3.** Single-cell HspXp-ECFP intensity, DCS 30 ug/mL.
M. smegmatis Expressing HspXp-ECFP in Responses to 30 ug/mL D-Cycloserine

Figure 4.4. Image frames showing single M. smegmatis cells expressing HspXp:ECFP in response to 30 ug/mL D-Cycloserine.

Figure 4.5, below, shows a similar drug treatment experiment in which cells were exposed to 100 ug/mL of DCS for 13 hours. Of note in Figure 4.3 above and in Figure 4.5 below is the fact that cells did not express ECFP fluorescence in the absence of drug; this is contrast to a previous report in which spontaneous pulses of the protein KatG were
observed in steady-state conditions [37]. While we refrain from assigning a mechanism underlying these pulses of transcriptional activity at the HspX promoter, it is likely that they are indicative of activation of some stress-response pathway within these cells.

![Figure 4.5. Single-cell ECFP fluorescence traces in response to 100 ug/mL D-Cycloserine.](image)

Our MATLAB image-processing pipeline was expanded to include peak-finder functionality for processing of single-cell ECFP fluorescence traces. Representative single-cell fluorescence traces are shown in Figure 4.6 below, showing that several of these cells exhibited peaks of fluorescence intensity over the course of the time-lapse experiment. Cell lengths are plotted in these graphs as well so that division events can be seen. From these single-cell traces, population-wide statistics about ECFP pulses can be obtained and compared across experimental conditions.
Figure 4.6. Single-cell ECFP fluorescence traces in response to 30 ug/mL DCS.

Single-cell cell length and growth rate measurements were also obtained for these experiments, and it was found that increased concentration of DCS resulted in a more rapid cessation of growth in the population (Figures 4.7-4.12). More cells grew while under drug treatment at 30 ug/mL than 100 ug/mL DCS as is apparent when comparing Figures 4.7 and 4.8. Resumption of growth after DCS removal occurred in fewer cells at an increased concentration of DCS, apparent in Figures 4.9 and 4.10 showing average growth rate (microns/hr) in the population.
Figure 4.7. Single-cell length measurements under DCS treatment (30 ug/mL).

Figure 4.8. Single-cell length measurements under DCS treatment (100 ug/mL).
Figure 4.9. Single-cell growth rates, DCS 30 ug/mL. Green lines, single cells; black line, population average.

Figure 4.10. Single-cell growth rates, DCS 100 ug/mL. Green lines, single cells; black line, population average.
To explore the functional significance of pulsatile HspX expression in single cells, propidium iodide staining was performed at the conclusion of each experiment and live/dead statistics were quantified for all cells. Cells were grouped by number of fluorescence pulses, and the odds of survival (i.e. the probability of survival divided by the probability of death) were calculated as a function of the number of fluorescence pulses observed. At DCS concentrations of both 100 \( \mu \)g/mL and 30 \( \mu \)g/mL we found that cells that pulsed were more likely to die than cells that did not pulse (Figure 4.11), with p-values of 0.16 and 0.09 respectively (one-tailed Fisher's exact test). Increased statistical power (i.e. higher numbers of cells) is needed to confirm this result; statistics were generated using 52 and 42 cells respectively. Figures 4.12 and 4.13 show that for a given drug treatment, pre- and post-drug growth rates are similar between persisters and non-persistent cells. Finally, Figures 4.14 and 4.15 show that post-drug, but not pre-drug, growth rates are different between cells that pulse and cells that do not pulse.

![Odds of survival vs. Number of Peaks](image)

Figure 4.11. Odds of cellular survival vs. number of HspXp-ECFP fluorescence peaks.
Growth Rates Before and After Drug Addition, DCS 30 μg/mL

Figure 4.12. Single-cell growth rates pre- and post-addition of 30 μg/mL DCS. Red histograms, non-surviving cells. Green histograms, persister cells.

Growth Rates Before and After Drug Addition, DCS 100 μg/mL

Figure 4.13. Single-cell growth rates pre- and post-addition of 100 μg/mL DCS. Red histograms, non-surviving cells. Green histograms, persister cells.
Pre-Drug Growth Rates

Pulsers
Average: $0.19 \pm 0.07 \text{ um/hr}$

NonPulsers
p-value: 0.366
Average: $0.20 \pm 0.14 \text{ um/hr}$

Figure 4.14. Growth rates pre-drug addition for pulsing vs. non-pulsing cells.

Post-Drug Growth Rates

Pulsers
Average: $0.04 \pm 0.05 \text{ um/hr}$

NonPulsers
p-value: 0.00
Average: $0.08 \pm 0.06 \text{ um/hr}$

Figure 4.15. Growth rates post-drug addition for pulsing vs. non-pulsing cells.
Taken together, these results show several intriguing and interconnected observations about the behavior of *M. smegmatis* cells under antibiotic stress. First is that *M. smegmatis* cells respond to DCS by slowing growth and by ‘pulsing’ transcription of the HspX promoter. Second is that higher DCS concentrations increase the proportion of cells that pulse, and hasten the response time of pulsing cells. Third, and perhaps most, interesting is that HspX pulsing is correlated with cell fate under DCS treatment. But what is the functional significance of HspX pulsing, and why is it negatively correlated with cellular survival? As discussed above, DosR is the strongest transcriptional regulator of HspX, and *M. smegmatis* cells that are exposed to stresses such as hypoxia, NO, and CO exhibit a dormancy response that is analogous to that of MTB [115]. However, in a study of MTB persisters that screened for DCS-tolerant cells, DCS was not found to induce DosR transcription although the sensor kinase DosS was upregulated [5]. Though more work is needed to elucidate the mechanistic basis linking HspX pulsing to cellular survival under DCS treatment, several hypotheses shed light on this open question. HspX might be indicative of a stress response pathway that is initiated in the pulsing cells; these cells may be more highly sensitive to DCS due to some physiological parameter (e.g. cell age) or their internal state (e.g. a higher number of some cellular component linked to stress). HspX pulsing could either be indicative of an adaptive response, or could conceivably be in fact counterproductive or maladaptive (akin to a drowning person thrashing wildly). To resolve this question, DosR could be exogenously induced either using inducer chemicals (e.g. the nitric oxide donor S-nitrosoglutathione) [116] or by placing DosR under control of a constitutively-expressed promoter [117]. The purpose of
this would be to artificially induce HspXp-ECFP pulsing in high numbers of cells and to determine if this would result in increased or decreased susceptibility to DCS treatment. Another hypothesis stems from recent work showing ‘altruistic’ behavior in microbes, whereby some cells, at a fitness cost to themselves, produce molecular signals or other molecules that confer drug tolerance to other members of the population [118, 119]. This could be tested using the methods of inducing DosR described above, and co-culturing JK306H cells with wild-type *M. smegmatis* cells to determine if HspX-ECFP pulsing in JK306H cells led to increased survival of wild type *M. smegmatis*. Future work will address these questions and build on the results presented here, as discussed in Chapter 5.

**Section Two: Cellular Responses to Hypoxia**

As described in Chapter 1, hypoxia has been identified as a key host-induced stress on MTB *in vivo* [8]. MTB initiates a well-characterized response to hypoxia, mediated in part by the transcription factor DosR, that involves coordinated transcriptional regulation of hundreds of genes and a host of metabolic changes [8-12]. The hypoxic induction of the DosR regulon is notable for its strength and speed, which suggest a critical functional role for the regulon in MTB’s hypoxic adaptation. In vitro, hypoxia induces a state of non-replicating persistence (NRP) in MTB that closely resembles the state of the organism in human infection; critically, MTB in hypoxia-induced dormancy exhibits increased tolerance to several classes of drugs [13-15]. Adaptations of MTB to hypoxia are therefore likely to play a major role in MTB pathogenesis, and may modulate drug sensitivity *in vivo*. 
To explore the single-cell behavior of *Mycobacteria* in a hypoxic microenvironment, we built a “hypoxic chamber” on a microfluidic chip based on a recently reported device architecture [120] that incorporates large (50x200 micron) gas channels adjacent to the on-chip cell trap. These channels are perfused with nitrogen during the course of an experiment, which purges the on-chip microenvironment of oxygen due to the high gas permeability of PDMS. To validate our ability to control oxygen tension on chip, we used two methods. First, we employed Ruthenium-coated glass cover slips, which fluoresces in the absence of oxygen [121, 122]; second, we built a hypoxia-inducible fluorescent strain of *E. coli* to monitor single-cell hypoxic induction in real-time [123]. Ruthenium fluorescence was measured in known environments of anoxic (0%) and ambient (20%) oxygen concentrations (Figure 4.16). The oxygen-sensitive *E. coli* nar promoter was fused to ECFP and cultured in the microfluidic hypoxia device (Figure 4.17) to further validate our ability to control oxygen tension on-chip while maintaining culture conditions amenable to live cells, and to monitor hypoxic response in single cells. The nar promoter is involved in anaerobic metabolism [31] and we saw uniform, strong induction of nar:ECFP within 30 minutes of oxygen displacement in the microfluidic device, which is on the order of one *E. coli* cell doubling time (20 minutes). These preliminary studies lay the groundwork for future analogous studies in *M. smegmatis*, specifically to address the question of whether hypoxic conditions confer a drug-tolerant state in the organism and whether this state can be observed and quantified in single cells in real time.
Figure 4.16. Ruthenium fluorescence within a microfluidic 'hypoxia chamber.'

Figure 4.17. Induction of the nar promoter in *E. coli* in response to hypoxic conditions in a microfluidic device. Oxygen displacement began at time $t=0$. Time in minutes.
CHAPTER FIVE: Conclusions and Future Directions

This chapter summarizes the results reported in this dissertation, and briefly outlines future directions for further studies of single *Mycobacteria* cells. The microfluidic live-cell imaging platform we developed, in conjunction with our automated image processing pipeline, enabled us to study *Mycobacteria* species at the single-cell level, in order to investigate dynamic phenomena (e.g. gene regulation) as well as cell-to-cell heterogeneity and its relationship to drug tolerance. These are related but disparate phenomena and we thus obtained diverse data about gene expression, drug tolerance, and cell growth and morphology under various conditions. Each of these datasets suggest directions for further study, which could lead to more comprehensive insights about processes such as the dormancy adaptation of MTB. Additionally, all of the work presented here was performed using *M. smegmatis*, but future work in MTB itself or in the attenuated *M. bovis* vaccine bacillus (BCG) would enable comparisons of any differences between these organisms, and would serve to enable more direct measurements of MTB biology. These experiments are technically challenging to perform due to the need to have all equipment in biosafety level 3 facilities, as well as the 24-hour doubling time of MTB and BCG as compared to 4 hours for *M. smegmatis*. However, recent reports of single cell studies in MTB have emerged [50], and as the fields of microfluidics and single-cell systems biology mature these experiments will become more common. In general the future directions discussed below could be performed in *M. smegmatis* first as a proof of concept and then adapted to BCG or MTB,
or if a microfluidic time-lapse imaging platform was installed in a BSL3 facility, this work could directly be performed in MTB itself.

Section One: DosR Expression in Response to Hypoxia and Other Stresses

Our results with the *E. coli* nar promoter show that the microfluidic platform described in this work can be used to generate controlled hypoxic environments on-chip. This platform could be used to study DosR transcriptional activation and/or regulon induction in hypoxia using *M. smegmatis* or MTB. Remaining work needs to be done to more accurately calibrate the system for fine control, especially considering that MTB has been shown to enter two different stages of non-replicating persistence as the oxygen tension falls below 1% and 0.06% oxygen saturation respectively [124-126]. Microfluidic platforms offer for the first time the ability to study at the single-cell level the non-replicating persistence of MTB in response to hypoxia. Because DosR is thought to act as a bistable switch due to its positive autoregulation, subpopulations of dormant and non-dormant cells might co-exist in culture as oxygen tension decreases from ambient saturation down to <1%; the fraction of cells entering dormancy may well be a function of oxygen tension, and this could be probed using the single-cell methods described here. For this work the DosR promoter and/or the DosR protein should be fused to a fluorescent protein so that transcriptional activation of DosR can be measured; most interesting would be if the HspX promoter were simultaneously monitored using a second color fluorescent protein construct, so that differences in DosR and HspX induction could be quantified in single cells. DosR expression is likely to be correlated with diminished
single-cell growth rates in hypoxic conditions, and this could also be shown directly using the single-cell platform. Additionally, the hypoxic response of DosR knockout strains of *M. smegmatis* and/or MTB could be analyzed at the single-cell level using this platform. Finally, drug tolerance of Mycobacterial cells could be investigated in the hypoxic microenvironment, which would serve as an *in vitro* model of host-like conditions. It would be interesting to determine whether induction of the DosR regulon is correlated with increased drug tolerance in these experiments, as it has been reported that DosR regulon induction *per se* is not a key mediator of drug tolerance *in vivo* [31].

**Section Two: Further Investigation of the MTB Regulatory Network**

We showed direct evidence of gene regulation for two members of the KstR regulon, and used this both as validation of a synthetic oscillator construct and as proof of concept of this technique in Mycobacteria. To more comprehensively understand the function of KstR or indeed any regulator in the MTB regulatory network, these studies should be extended to include more members of the TF’s regulon. Additionally, this single-cell analysis platform could be used to directly validate feed-forward loops and other network motifs that have been predicted computationally; these would require two- or three-color fluorescence measurements simultaneously but would potentially lend very powerful insights to ongoing efforts at regulatory network reconstruction [24]. Similarly, some of the putative links between TFs determined by ChIP-seq could be directly probed using multi-color fluorescence constructs and inducible promoter systems. The results
presented here focused on TF-gene target measurements, but providing direct evidence of regulation at the level of TFs would be more directly applicable in answering some of the questions that have arisen from current network reconstruction efforts. These include linking the regulators DosR, Rv0081, KstR, and Rv3024, which were predicted in [24] to form a core subnetwork linking the hypoxic response adaptation with cholesterol and lipid metabolism pathways. Finally, as described in Section One of this chapter, an interesting extension of this work would be to perform these gene regulation studies in MTB itself or in M. bovis BCG, either recapitulating the work we presented in M. smegmatis or investigating other TFs and pathways. This work could also incorporate the addition of specific metabolites to the on-chip environment, e.g. using cholesterol to de-repress KstR transcription, thus using the metabolites themselves as ‘inducers’ and observing specific TF or gene induction in response.

Section Three: Studying Mycobacterial Infection of Macrophages on-Chip

Finally, the microfluidic platform described here could be incorporated with macrophage culture on-chip and used for the long-term imaging of MTB infection processes. Several groups have used microfluidic devices to obtain measurements from living macrophages, demonstrating the technical feasibility of on-chip macrophage culture [127, 128]. Fluidically isolated seeding chambers were used to perform control and experimental assays in parallel, and on-chip valves were used to sequentially perform cell stimulation, permeabilization and analysis steps. Critically, macrophages in the microfluidic device did not express environmental-stress proteins in response to shear
rates produced by fluid flow. Fluorescent tags could be used to monitor gene expression changes in the macrophages themselves, as well as the mycobacterial cells, in response to infection [129]. This technique would represented one step closer towards true in vivo single-cell biology and could be used for many different single-cell analyses, e.g. to test drug responses in single cells in the macrophage environment or to examine DosR transcriptional induction in single cells at different physical locations within the macrophage. It is also possible that gene expression and/or morphological characteristics of mycobacterial cells would be largely similar for all cells within the same macrophage, but different for cells residing in different macrophages; this would suggest variability within the macrophage environment. With these experiments as with the other future directions outlined in this Chapter, the power of these techniques would be to observe interesting correlations between single-cell phenotypes and behavior, e.g. DosR induction and antibiotic tolerance, as this type of investigation truly requires single-cell analysis and has thus been unexplored until today. The broader aim of this work is to better elucidate our understanding of MTB biology, specifically with regard to pathogenesis and infection, in the hope that this understanding will lead to the development of new tools for combating this ubiquitous and devastating human pathogen. Systems biology at the single-cell level is truly a fertile ground for future research, and it will be exciting to see what new insights come of these techniques.
APPENDIX 1: SAMPLE MATLAB CODE

This function, drawGrowthRate.m, draws single-cell growth rates, taking as input the cell array structures CellFluore and ExptData which contain the cell meshes drawn by the MicrobeTracker program as well as all experimental parameters.

```matlab
function []=DrawGrowthRate2(varargin)

exptData = evalin('base','exptData');
CellFluore = evalin('base','CellFluore');
T = exptData.T;
figure('Name','Growth','NumberTitle',on', 'Position',[1,1,1920,1080],'Units','pixels');
numberOfLines = size(CellFluore.Filterlength,2);
dupTiming = CellFluore.duplicateTiming;

% Make the colors
finalColors = zeros(numberOfLines,3);
for i = 1:numberOfLines
    finalColors(i,:) = [rand, (0.4)*rand + 0.2, rand];
end
finalColors = finalColors*0.97;

%Get the right cells
GoodCells = CellFluore.CellNum;

%Get cell length and d(length)/dt information
tmp=CellFluore.Filterlength;
tmpDiff = diff(tmp);
timeDiff = T(2:size(CellFluore.Filterlength,1));

% Don't show doubling events in the growth plot
for i = 1:length(GoodCells)
    for timepoint = 1:length(timeDiff)
        if(dupTiming(timepoint,i) == 1)
            tmp(timepoint,i) = NaN;
        end
    end
end

hold on
for i = 1:length(GoodCells)
    if GoodCells(i) == 0
        line(T,tmp(:,(i)),...'
            Color',finalColors(i,:),'
            LineWidth',0.5,'
            LineStyle','-');
    end
end

minMax = ylim;
minMax(2) = minMax(2)*0.995;
ylim([minMax(1) minMax(2)]);

%Drug Info
if ~isempty(exptData.onTime)
drugTimeArray = [exptData.onTime, exptData.offTime];
drugAbbrev = strtok(exptData.drugName, ', ');
drugAdded = [drugAbbrev, 'added'];
drugRemoved = [drugAbbrev, 'removed'];
line(drugTimeArray,'k-','k-',[drugAdded, drugRemoved]);
end

%Hypoxia Info
if ~isempty(exptData.hypoxOn)
hypoxTimeArray = [exptData.hypoxOn, exptData.hypoxOff];
line(hypoxTimeArray,'k-','k-',['Hypoxia','']);
end
```
% Construct Title
titleText = ['Cell Length', ',', '...',
            'exptData.drugName', ',', 'ug/mL'];

title(titleText, 'FontSize', 16, 'interpreter', 'latex');
xlabel('Hours', 'FontSize', 16, 'interpreter', 'latex');
ylabel('Cell Length (microns)', 'FontSize', 16, 'interpreter', 'latex');

goodIndices = [];
for i = 1:length(GoodCells)
    if GoodCells(i) ~= 0
        goodIndices = horzcat(goodIndices,i);
    end
end

% This takes tmpDiff from only the cells we care about
tmpDiff = tmpDiff(:,goodIndices);

% Disregard final frame
for i = length(averagetmpDiff)-1:length(averagetmpDiff)
    averagetmpDiff(i)=0;
end

% This filters drastic changes in tmpDiff
for i = 1:length(tmpDiff(:,1))
    for j = 1:length(tmpDiff(1,:))
        if (tmpDiff(i,j))< -0.2
            tmpDiff(i,j)=0;
        elseif (tmpDiff(i,j))>1
            tmpDiff(i,j)=1;
        end
    end
end

% This smooths averagetmpDiff with a moving avg window of 3
windowSize = 3;
filterData = filter(ones(1,windowSize)/windowSize,1,averagetmpDiff);

% This finds the average growth rate
filterSingleCellTraces = zeros(size(tmpDiff));
for i = 1:length(tmpDiff(:,1))
    filterSingleCellTraces(:,i) = filter(ones(1,windowSize)/windowSize,1,tmpDiff(:,i));
end
n = length(filterSingleCellTraces(:,1));
f = fft(filterSingleCellTraces(:,1));
f(n/2+1:5:n/2+5) = zeros(10,1);
filterSingleCellTraces(:,i) = real(ifft(f));

averageSingleCell = mean(filterSingleCellTraces,2);
% Draw avg growth rate
figure
hold on
beta = length(T);
framesPerHour = 4;

% Green curves
for i = 1:length(goodIndices)
    plot(T(2:beta),framesPerHour*filterSingleCellTraces(:,i),'LineWidth',1,'Color',[0.5 0.95 0.7]);
end
numCellsPlotted = strcat(num2str(length(goodIndices)),' Cells Plotted');

% Black curve
plot(T(2:length(T)),framesPerHour*averageSingleCell, 'LineWidth',4,'Color',[0.1 0.1 0.2], 'LineStyle', '-');
ylim([-0.1 2]);

% Drug Info
if ~isempty(exptData.onTime)
drugTimeArray = [exptData.onTime, exptData.offTime];
drugAbbrev = strtok(exptData.drugName, ' ');
drugAdded = [drugAbbrev, 'added'];
drugRemoved = [drugAbbrev, 'removed'];
vline(drugTimeArray,{'k-.','k-.'},{'drugAdded', drugRemoved});
end

% Hypoxia Info
if ~isempty(exptData.hypoxOn)
hypoxTimeArray = [exptData.hypoxOn, exptData.hypoxOff];
vline(hypoxTimeArray,{'k-.','k-.'},{'Hypoxia',' '});
end

% Construct Title
titleText = ['Cell Growth Rate (microns/hr),', ' ', ..., exptData.drugName, ' ug/mL'];
title(titleText, 'FontSize', 16,'interpreter','latex');
xlabel('Hours', 'FontSize',16,'interpreter','latex');
ylabel('Cell Growth Rate, um/hr', 'FontSize',16,'interpreter','latex');
hline(0, 'r-');
text(max(xlim)*0.25,max(ylim)*0.95, numCellsPlotted, 'FontSize', 15);
BIBLIOGRAPHY


VITA