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Publication Date
2012

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA, SAN DIEGO

On the synchronization of synthetic genetic oscillators in single cells and colonies

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Octavio Mondragon-Palomino

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2012
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Chair

University of California, San Diego

2012
DEDICATION

Dedico este trabajo a mi familia y amigos en México. Por la prolongada ausencia.
# TABLE OF CONTENTS

Signature Page .............................................................. iii

Dedication ................................................................. iv

Table of Contents ......................................................... v

List of Abbreviations ...................................................... vii

List of Figures ............................................................ viii

Acknowledgements ....................................................... xi

Vita .............................................................................. xiii

Abstract of the Dissertation ............................................ xv

Chapter 1  Introduction ................................................... 1

Chapter 2  Microfluidic Technology for Quantitative Biology .... 6
   Flow of fluids and mass transfer in microscopic scales ........ 7
   The movement of fluids in micro-channels ..................... 8
   The transfer of mass in micro-channels ....................... 10
   Recent applications of microfluidics in quantitative biology .. 12
   Microfluidics for single-cell microbiology .................... 13
   Bacterial group behavior ........................................... 18

Chapter 3  Entrainment of a Population of Synthetic Genetic Oscillators .... 21
   Introduction .......................................................... 21
   The physics of entrainment and modeling results ........... 22
   Experimental results .............................................. 23
   Discussion and modelling ....................................... 28
   Conclusions and future work ................................... 31
   Acknowledgements ................................................. 32

Chapter 4  A Synchronized Quorum of Genetic Clocks ............ 33
   Introduction ........................................................ 33
   Synchronized genetic oscillators ............................... 35
   Spatiotemporal Dynamics ....................................... 39
   Quantitative modeling .......................................... 40
   Emergence ......................................................... 42
   Perspective and outlook .................................... 44
   Acknowledgements .............................................. 45
LIST OF ABBREVIATIONS

A.U. ..................................................... arbitrary units
bp ....................................................... base pair
DES ..................................................... discrete element simulations
DNA ..................................................... deoxyribonucleic acid
E. coli .................................................. *Escherichia coli*
Eq. ..................................................... equation
fig. ..................................................... figure
FL. ..................................................... fluorescence
FP ..................................................... fluorescent protein
FSC ...................................................... forward scatter
GFP ..................................................... green fluorescent protein
hrs. ..................................................... hours
IPTG .................................................. isopropyl β-D-1-thiogalactopyranoside
kb ..................................................... kilobase
LB ..................................................... lysogeny broth
mRNA ................................................ messenger ribonucleic acid
OD ..................................................... optical density
ODE ................................................... ordinary differential equation
PCR ................................................... polymerase chain reaction
PDMS ................................................ poly(dimethylsiloxane)
PIV .................................................... particle image velocimetry
Re ..................................................... Reynolds number
S. cerevisiae ......................................... *Saccharomyces cerevisiae*
UV ..................................................... ultraviolet
yeGFP ............................................. yeast-enhanced cyan fluorescent protein
YFP .................................................. yellow fluorescent protein
%w/v ............................................. percent weight per volume (concentration)
LIST OF FIGURES

Figure 2.1: Definition of pole age in *E. coli* ........................................... 14
Figure 2.2: A linear colony of bacteria grows in a microcavity ..................... 15
Figure 2.3: Microfluidic setup for observing *Mycobacterium* cells and the pattern
of deterministic variability in their lineage ........................................... 19
Figure 2.4: Bulk and single-cell measurements of a wave of chemotactic bacteria
........................................................................................................ 20

Figure 3.1: Overview of the time-lapse fluorescence experiments ................... 24
Figure 3.2: Probability distributions of the relative phase ............................. 25
Figure 3.3: Probability distributions of the period ........................................ 27
Figure 3.4: Computational modeling shows that extrinsic sources are the dominant
contribution to variability ................................................................. 30

Figure 4.1: Synchronized genetic clocks .................................................... 36
Figure 4.2: Dynamics of the synchronized oscillator under multiple microfluidic
flow conditions ................................................................................. 38
Figure 4.3: Spatiotemporal dynamics of the synchronized oscillators ............. 41
Figure 4.4: Modeling of synchronized genetic clocks ................................... 43

Figure 5.1: Overview of experimental results .............................................. 48
Figure 5.2: Stability analysis of the 2D continuum model ............................. 49
Figure 5.3: Space-time diagrams of the narrow-channel flow from discrete ele-
ments simulation .............................................................................. 52
Figure 5.4: Streaming patterns in a wide side trap from DES simulations ...... 54

Figure 7.1: Microfluidic device constructed to perform entrainment experiments 66
Figure 7.2: Comparison of the experimental and stochastic model probability dis-
tributions of the relative phase for *A* =0.075 ....................................... 67
Figure 7.3: Comparison of the experimental and stochastic model probability dis-
tributions of the period for *A* =0.075 ................................................. 68
Figure 7.4: Comparison of the experimental and stochastic model probability distributions of the relative phase for $A = 0.15$ ................................. 69
Figure 7.5: Comparison of the experimental and stochastic model probability distributions of the period for $A = 0.15$ ................................. 70
Figure 7.6: Comparison of the experimental and deterministic model probability distributions of the relative phase for $A = 0.075$ ................................. 71
Figure 7.7: Comparison of the experimental and deterministic model probability distributions of the period for $A = 0.075$ ................................. 72
Figure 7.8: Comparison of the experimental and deterministic model probability distributions of the relative phase for $A = 0.15$ ................................. 73
Figure 7.9: Comparison of the experimental and deterministic model probability distributions of the period for $A = 0.15$ ................................. 74
Figure 7.10: Plasmids for the synchronized oscillator strain TDQS1. ............... 77
Figure 7.11: Stable oscillations in microfluidic device ..................................... 78
Figure 7.12: Space-time plot of density of cells in Fig.4.3a experiment ............... 79
Figure 7.13: Microfluidic Devices constructed for this study ............................. 83
Figure 7.14: Synchronization of oscillations in spatially extended system with diffusion ................................................................. 85
Figure 7.15: Wave propagation in the spatially uniform system with different external AHL diffusion rates .................................................... 86
Figure 7.16: Space-time plot of the average exit velocity at the top edge of the trap, similar to Fig. 5.1d ......................................................... 102
Figure 7.17: Schematic diagrams of side trap experiments and the corresponding space-time plots ................................................................. 104
Figure 7.18: Space-time plots for experiment in Supplementary Movie 4 ............ 105
Figure 7.19: Snapshot of a colony growing in the $\sim 1 \mu m$-high chamber .......... 106
Figure 7.20: Schematic view of the Jacobian matrix ...................................... 106
Figure 7.21: A local bifurcation analysis of the narrow channel flow ............. 107
Figure 7.22: Space-time diagrams for simulations of cell flows in wide side traps with different cell aspect ratios ............................................ 108
Figure 7.23: Snapshots at time $t = 30$ of the three simulations in Fig. 7.22 .... 109
ACKNOWLEDGEMENTS

If I made a diagram connecting all the people and the circumstances that made possible the completion of this work, it would probably end up looking like one of those hairballs so common in systems biology. And as it happens with the biology hairballs, in the end I would be missing half of the links and nodes, and have serious doubts about the rest. So as we do in the lab, I will draw the minimal network that made everything happen. First, with a big node for himself, comes Prof. Jeff Hasty, who opened the doors of his lab to me. I thank Jeff for the opportunity and for all the lessons on the real practice of science. I need another big node for Dr. Lev Tsimring, who always kept his door wide open to come and learn science from him or discuss a new project. I should also thank Lev for those summer parties, so eventful. My friend Tal Danino was all positive feedback. From him I learned I should not be shy about trying my own ideas in the lab. My collaborators Jangir Selimkhanov and Will Mather have their own part of the network for their hard, hard work. Everyone else in the lab, thanks for teaching me, making it enjoyable to come everyday to work and keeping the lab free of drama. Martin and Ivan have been great lab mates and at adding a touch of humor. Special recognition to the old guard, Scott, Natalie, Mike and Lee for setting the base of the lab I came to know and enjoy. Also I would like to acknowledge the undergrads, Alex Vo and Kristian Lorenzo, who worked with me for almost three years, helping me with microfluidics and giving me the opportunity to mentor them.

Outside the lab, my parents and my sister, although at the distance, were always my fundamental support, as well as my second mom, aunt Lulu, and the Lopez families in LA and San Diego. Finally, in her own heart-shaped and beating node, comes Annette with whom I sort all the ups and downs, past and future.


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ABSTRACT OF THE DISSERTATION

On the synchronization of synthetic genetic oscillators in single cells and colonies

by

Octavio Mondragon-Palomino

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2012

Jeff Hasty, Chair

Synthetic and systems biology propose the rational construction and alteration of the molecular networks that govern life processes, with the purpose of understanding how their architecture relates to the emerging dynamics and for the creation of novel functions. This approach is made possible by the tools of modern molecular biology and the quantitative insights of physical sciences. In this context, microfluidic technology has become the tool of choice for our investigations. Our work is greatly motivated by the prominent role that oscillations play in organizing the dynamics of all living things and by the observation of self-organization of bacterial colonies. In Chapter 2 the basic physics of fluid flow and mass transport in microfluidic devices are presented along with several examples of the application of this technology to quantitative biology. In Chapter 3 I discuss a study on the entrainment of biological clocks through the use of a bacterial synthetic oscillator. In Chapter 4 we discuss the construction and characterization of a genetic oscillator that produces group oscillations. Finally, in Chapter 5 we discuss the dynamic mechanical interactions that arise from the spatially constrained growth of bacteria.
Chapter 1

Introduction

Living organisms continuously experience changes in their physiology and behavior due to the expression of internal genetic programs and the regulated responses to interactions with other organisms and their physical environment. In single cells, these changes occur over a wide range of time scales. For instance, upon transient stimulation the response time of the chemotaxis system in the bacterium *Escherichia coli* is of the order of a few seconds (Segall et al., 1986). On the other hand, the process of aging in the wild-type Yeast *Saccharomyces cerevisiae* occurs over the lifespan of a cell, which can last from days to weeks (Breitenbach et al., 2011).

Without regard to their time scale, there are important biological processes that occur periodically (Kruse and Jülicher, 2005; Buzsáki and Draguhn, 2004). Relevant examples include bacterial cell division and the eukaryotic cell cycle (Kruse and Jülicher, 2005). In all vertebrates and a few microorganisms, these programs interact with another cyclic program known as the circadian clock (Lakin-Thomas and Brody, 2004; Bell-Pedersen et al., 2005). For example, in cyanobacteria the circadian clock drives incompatible chemical reactions to occur at different times, gates cell division and has the ability to be reset by the environmental cycle of light and darkness (Berman-Frank et al., 2001; Compaoré and Stal, 2010; Rust et al., 2011; Yang et al., 2010). Therefore, the circadian clock plays a special role because it is involved in keeping internal phys-
iological harmony, like the cell cycle does, while keeping organisms in tune with their surroundings.

In multicellular organisms timekeeping is achieved through a hierarchical system of circadian clocks (Panda et al., 2002; Dibner et al., 2010). The region of the human brain known as the suprachiasmatic nucleus (SCN) acts as the central pacemaker of the system, influencing the daily cycles of physical variables like temperature and blood pressure. Human behavior also receives important input from the circadian clock through the timed release of hormones like melatonin, which regulates our sleep patterns. Like in the clock of cyanobacteria, the phase of the SCN’s rhythms can be shifted by environmental signals in a process known as entrainment. Moreover, the SCN is able to process the phase information and pass it down to other regions of the brain and the body (Dibner et al., 2010). In fact, almost all the cells in the human body carry a functional circadian clock that is resettable by temperature and other physiological stimuli (Dibner et al., 2010; Stokkan et al., 2001; E et al., 2004). Nevertheless, only the cells of the SCN are capable of mutually synchronizing their cadence to perform collectively as a central pacemaker. Therefore, the success of the circadian system in organizing multicellular physiology is based on the ability of individual clocks all over the organism to “listen” to each other and to shift their beating to exogenous inputs.

The molecular networks that sustain circadian rhythms in single cells of different organisms are known in great detail (Zhang and Kay, 2010; Harmer, 2009). In spite of this, our understanding of higher functions like synchronization and entrainment are still incomplete. This is due to the daunting complexity of the networks and to the challenges posed by experimentation with higher organisms (Zhang and Kay, 2010; Harmer, 2009; Sellix et al., 2010; Wenden et al., 2012).

The synthetic and systems biology approach proposes that intricate biological networks can be fragmented into smaller subnetworks that are easier to understand in a quantitative manner (Alon, 2007a). In particular, synthetic biology postulates that a better way to understand the principles behind these small modules is by constructing them, and that the function of the larger network can be recovered by a systematic recon-
struction on the basis of well understood network motifs (Hasty et al., 2002; Sprinzak and Elowitz, 2005; Nandagopal and Elowitz, 2011). Many small genetic modules have been built using standard molecular biology techniques (Gardner et al., 2000; Elowitz and Leibler, 2000; You et al., 2004; Basu et al., 2005; Kobayashi et al., 2004; Austin et al., 2006; Tabor et al., 2009; Friedland et al., 2009). Given the preminence of oscillators in biology, physics and engineering, a natural testing field for synthetic biology is the development of biological oscillators (Elowitz and Leibler, 2000; Atkinson et al., 2003; Fung et al., 2005; Stricker et al., 2008a; Tigges et al., 2009, 2010). An important proportion of the work I present in this dissertation (Chapters 3 and 4) is related to the study of the fundamental processes of entrainment and synchronization of genetic clocks using a combination of synthetic biology, quantitative experimentation and computational simulations (Danino et al., 2010; Mondragon-Palomino et al., 2011).

Our experimental approach to studying the dynamics of synthetic genetic oscillators relies on the expression of the green fluorescent protein (GFP) or its variants (Tsien, 1998). When the production of GFP is driven by a synthetic genetic program inside cells, the intracellular amount of the protein provides a measure of the activation state of the transcriptional promoters of the program. We use this readout to infer the dynamics of the synthetic genetic network. To measure the amount of GFP present in cells over time, the protein is optically excited and the fluorescence signal is recorded. Standard fluorescence microscopy is well suited for this (Young et al., 2011). However, the practice of microscopy has been based on techniques to grow small groups of cells in static conditions and for relatively short periods of time, limiting the study of cellular dynamics. To develop the work presented in this dissertation, I have created microfluidic technology to obtain long and abundant fluorescence trajectories in single cells and bacterial colonies (Danino et al., 2010, PMID: 20090747; Mondragon-Palomino et al., 2011). The first Chapter provides an introduction to the basic concepts of microfluidics and reviews several outstanding examples of the application of this technology towards the study of biological phenomena at multiple temporal and spatial scales.
In Chapter 3 is described our work on the entrainment of genetic oscillators (Mondragón-Palomino et al., 2011). Until now, the development of synthetic oscillators has been aimed at understanding how the interplay of different design features found in natural clocks, such as positive and negative feedback and delay, produce robust rhythms. One aim of our work was to show that synthetic constructs can be used as simplified model systems for the quantitative study of dynamic phenomena like entrainment. As a first step, we thoroughly characterized the dynamics of a synthetic oscillator that is forced by an external periodic signal, in analogy with the resetting of native clocks by changing environmental inputs. Such a detailed picture of entrainment has not been accomplished in native systems due to the intricacy of the underlying networks and experimental difficulties. In this work we also looked at the role positive feedback has on the sensitivity of genetic clocks and speculate about the implications this may have in natural settings.

In Chapter 4 are discussed our results on the engineering and characterization of population-wide oscillations of gene expression in E. coli (Danino et al., 2010). Multiple examples of coordinated populations of organisms or cells can be found in Nature (Glass, 2001). An aspiration of synthetic biology has been to engineer the coordinated oscillations of well understood, albeit noisy, synthetic oscillators (McMillen et al., 2002; Garcia-Ojalvo et al., 2004). Soon after the publication of the repressilator, McMillen et al. proposed that independent self-sustained oscillators can be synchronized if each oscillator cell carries a coupling module that relays a chemical signal between a cell and its neighboring oscillators. This work discusses the emerging spatio-temporal dynamics of a clock that merges the coupling and oscillator functions in a single module.

Finally, in Chapter 5 we discuss the dynamics of the mechanical interactions of bacteria that live in microcavities (Mather et al., 2010). Bacteria often build into dense communities, called biofilms, where the interactions between cells and with their environment become important. Moreover, microbes tend to grow in cavities and crevices where they find shelter from environmental insults. When bacteria grow in such micro-
scopic constrictions, physiological heterogeneities may arise across the cellular population due to gradients of nutrients and cellular waste. We discovered that when the height of microfluidic chambers matches the width of *E. coli* cells, the spatial arrangement of cells is dynamic. This work discusses our findings in different cavity geometries and the quantitative analysis of our observations.
Chapter 2

Microfluidic Technology for Quantitative Biology

Tracking any biological process over time unavoidably leads to the observation that genetically identical cells in identical environments display considerable variability (McAdams and Arkin, 1999). Consequently, the quantitative biologist that is interested in the dynamics of the cell is often confronted by multiple experimental challenges. First, the single-cell traces that are necessary to gain mechanistic insight into cellular networks cannot be obtained from bulk measurements that average-out the individual behavior. The second challenge is obtaining abundant single-cell traces that provide significant statistics, as opposite to a few anecdotal trajectories. Additional obstacles arise if the phenomenon of interest results from the coordinated action of many cells. This may require the ability to observe entire populations without sacrificing single-cell resolution and creating the conditions for cellular coordination.

These challenges have been addressed separately. The need for abundant data is usually satisfied by flow cytometry (Shapiro and Leif, 2003), which is a standard technology to measure the fluorescence and size of single cells, but is limited to the acquisition of snapshots: a particular cell cannot be followed over time in a cytometer. Single-cell traces can be obtained using a microscope. However, practical issues like
cellular motility, the exhaustion of nutrients, etc. can restrict the duration of experiments. On top of these issues, the standard techniques to maintain viable cell cultures in microscopy experiments, like agar pads and culture wells (Goldman, 2005), do not mimic the spatially-structured and changing environments that living organisms thrive in. The rapid development of microfluidic technology is contributing to the removal of these experimental limitations and to the transformation of biology into a quantitative science (Whitesides et al., 2001; Whitesides, 2006; ). In this Chapter I will briefly introduce the basic physics of microfluidics. The objective is to give the reader an intuitive understanding of the temporal and spatial scales of the transport of matter at the microscopic scale, starting from general physical laws. I believe this is necessary to have a rational understanding of the microfluidic designs used in our experiments and those of others. After this introduction I discuss several outstanding examples that illustrate how fluidic technology is currently used to perform measurements of unprecedented quality in single cells and entire colonies.

An exhaustive and detailed discussion on the design and soft lithography techniques for the fabrication of a microfluidic device, as practiced in the Hasty Lab, is discussed elsewhere (Ferry et al., 2011).

**Flow of fluids and mass transfer in microscopic scales**

Microfluidics deals with the manipulation of very small quantities of fluid (10^{-9}-10^{-18} L) that are subject to definite external forces within channels whose dimensions are comparable to the width of a human hair (10 × 10^{-6}-200 × 10^{-6} m). The physics of fluids and mass transport in such small constrictions does not follow the intuition we gain from our everyday macroscopic experience (*i.e.* washing your hands, preparing a cup of tea). Accordingly, in the first two section of this chapter we will review the basic consequences of the miniaturization of fluid flows.

A key advantage of soft lithography microfluidics is that it allows the creation of features and chambers that are of the same order as cellular length scales. As will be
appreciated throughout this Dissertation, the physical laws discussed above can be used to rationally design microfluidic devices that meet the desired rates of flow and mass transfer to host colonies of any size, from a single cell to millions of them (Cookson et al., 2005; Mather et al., 2010; Danino et al., 2010, PMID: 20090747; Wang et al., 2010; Mondragón-Palomino et al., 2011; Prindle et al., 2011).

The movement of fluids in micro-channels

The movement of fluids is governed by the Navier-Stokes (NS) equations:

$$\rho [\partial_t v + (v \cdot \nabla)v] = -\nabla p + \eta \nabla^2 v + \rho g,$$

(2.1)

where $v(\mathbf{r}, t)$ is the velocity field, $p$ is the pressure, $\rho$ is the mass density, $\eta$ is the dynamic viscosity and $g$ is the gravitational acceleration.

Depending on the velocity of the flow and the dominant length scale of the problem, each term in NS equations has a different weight. One way of making these weights evident, is by removing the physical dimensions from the equations. To do that we will consider a rectangular channel where the characteristic velocity $v_o$ and length $l_o$ are the average velocity of the fluid and the smallest dimension of the channel, the height, for example. From these characteristic dimensions, the intrinsic scales for pressure $p_o=l_o/\eta v_o$ and time $t_o=l_o/v_o$ follow. Variables without dimensions are obtained by normalizing the physical variables by their characteristic dimensions:

$$x^* = x/l_o$$

(2.2)

$$v^* = v/v_o$$

(2.3)

$$p^* = p/p_o$$

(2.4)

$$t^* = t/t_o.$$  

(2.5)

Substituting the physical variables by the dimensionless variables in 2.1 and assuming gravitation does not have a net effect on the flow gives:

$$\rho \left[ \frac{v_o}{l_o} \partial_t v^* + \frac{v_o^2}{l_o} (v^* \cdot \nabla^*)v^* \right] = -\frac{p_o}{l_o} \nabla^* p^* + \frac{\eta v_o}{l_o^2} \nabla^* v^*,$$

(2.6)
where $\partial_t^* = t_o \partial_t$ and $\nabla^* = l_o \nabla$. Factorizing the constants in 2.6 yields:

$$Re[\partial_t^* v^* + (v^* \cdot \nabla^*) v^*] = -\nabla^* p^* + \nabla^{*2} v^*. \quad (2.7)$$

The constant $Re$ is the well known Reynolds number and is defined as $Re \equiv \rho v_o l_o / \eta$.

Typically, in microfluidic devices $v_o \sim 100\mu m/s$ and $l_o \sim 100 \mu m$, making $Re \ll 1$ and therefore the viscous dissipation of momentum $\nabla^{*2} v^*$, is of a smaller order than $Re(v^* \cdot \nabla^*) v^*$. Then, in steady state ($\partial_t^* = 0$ and $\nabla^* = 0$), the NS equations are reduced to a linear Stokes equation:

$$0 = -\nabla p + \nabla^{*2} v, \quad (2.8)$$

where the forces due to pressure gradients balance with the dissipation of momentum.

It is necessary to note here that the NS equations for the idealized rectangular channels in microfluidic devices reduce, in steady-state, to the same form as the Stokes equations. The reason for this is that the velocity field is translation invariant (if the direction of the flow is $x$, then $\partial_x v = 0$) and then the convective term $(v \cdot \nabla) v$ in the NS equations is identically zero. Therefore in the limit where $Re \ll 1$, the solution to the flow in an infinite rectangular channel can be used as a good approximation to the flow in a microfluidic channel. The solution to the idealized flow (Bruus, 2008), known as Poiseuille flow is:

$$v_x(y, z) = \frac{4 h^2 \Delta p}{\pi^3\eta L} \sum_{n, odd} \frac{1}{n^3} \left[1 - \frac{\cosh(n\pi y h)}{\cosh(n\pi w / 2h)}\right] \sin(n\pi z / h). \quad (2.9)$$

The flow rate $Q$ is found by integrating the velocity field over the cross section of the channel. For a rectangular channel with height $h$, width $w$ and length $L$, it can be shown that:

$$Q \approx \frac{h^3 w \Delta p}{12\eta L} \left[1 - 0.630 \frac{h}{w}\right], \quad (2.10)$$

in the approximation where $h < w$. Rewriting this result as

$$\Delta p = \frac{12\eta L}{1 - 0.630 h/w} Q, \quad (2.11)$$
and defining the hydraulic resistance as \( R_h = 12\eta L/(1 - 0.630h/w) \) the relation between the volumetric flow \( Q \) and the pressure drop along a straight channel can be written in the compact form:

\[
\Delta p = R_h Q. \tag{2.12}
\]

This is known as the Hagen-Poiseuille (HP) law and has the same form as Ohm’s law for the flow of electric current \( I \) across a resistance of magnitude \( R \) due to a voltage difference \( V: \Delta V = RI \). The hydraulic resistance is related to the viscous dissipation of momentum in analogy to the dissipation of energy by the electric resistance. The HP law along with the principles of conservation of mass and energy can be used to find the flows in a microfluidic device from the knowledge of the pressure drops and the hydraulic resistance (Bruus, 2008). Otherwise, if the the geometry of the channels does not allow the approximation \( h < w \) or details of the flow are sought, finite-elements computational tools like COMSOL can be used.

**The transfer of mass in micro-channels**

The microfluidic devices made in our lab consist of a set of channels "engraved" on the surface of a polymeric solid matrix that is bonded to a glass coverslip. The glass makes one side of channels and the polymer the other three.

Small molecules, like the nutrients for cells or the inducers of transcription, are transported in our microfluidic devices by the movement of the fluid (convection) and by the diffusion of these molecules down gradients of concentration. Another, often neglected, mode of molecular transport is the diffusion of gases through the polymeric matrix of the devices. Indeed, polydimethylsiloxane (PDMS) is, at the molecular scale, very porous and permeable to gases (Merkel et al., 2000). The surface of PDMS is also capable of adsorbing a wide range of molecules (Toepke and Beebe, 2006). Recent work takes advantage, sometimes unintentionally, of the permeability of PDMS to gases to deliver gaseous mixtures to cellular cultures and for the communication between bacterial colonies (Polinkovsky et al., 2009; Prindle et al., 2011).
The transport of a solute is described by the convection-diffusion equation; the concentration of the solute (number of molecules per unit volume) $c(r, t)$ changes according to:

$$\partial_t c + (v \cdot \nabla)c = D \nabla^2 c. \tag{2.13}$$

It is clear that convection and diffusion are at work all the time in microfluidic channels, and that they cannot be turned off at will. Nevertheless, it is possible to design the channels’ geometry to favor one mechanism over the other one. In the absence of flow, Eq. 2.13 reduces to the diffusion equation $\partial_t c = D \nabla^2 c$. Dimensional analysis of the diffusion equation relates the diffusion constant $D$ to the characteristic time $t_o$ and length $l_o$ for the change of the concentration $c$ leading to

$$D = \frac{l_o^2}{t_o}. \tag{2.14}$$

The constant $D$ determines how fast a substance diffuses down a concentration gradient. For biologically relevant molecules diffusion is slow, even at the microscopic scales found in microfluidic devices. For example, the typical diffusion constant for the sugars bacteria use as carbon sources is $D \sim 5 \times 10^{-10} m^2 s^{-1}$, which means that it takes approximately twenty seconds to diffuse over a distance of 100 microns.

If convection and diffusion are at work simultaneously it is possible to determine, from the timescales for diffusive and convective transport, which mechanism is faster. In the case of a very long channel with rectangular cross section of width $2w$, where the average flow is $v_o$ and the diffusivity of the solute is $D$, the time scale for lateral diffusion is of the order of $T_{diff} \sim w^2 / D$ and the time scale for convection in the direction of the flow over the same distance is simply $T_{conv} \sim w / v_o$. Therefore, the ratio of the diffusion time to the convection time is

$$Pé \equiv \frac{w^2 / D}{w / v_o} = \frac{wv_o}{D}. \tag{2.15}$$

This ratio is better known as the Péclet number ($Pé$) and is particularly relevant when researchers wish to mix two fluid phases. For example, consider a stream carrying a
solute runs parallel to a blank stream in a microfluidic channel of width $w$. Because the flow is laminar ($Re \ll 1$) and channels have smooth surfaces, the only mechanism for mixing is diffusion and, as a result, the length the two streams have to run in parallel to mix completely is $d_{mix} = v_0 t_{mix}$, where $t_{mix}$ is the time it takes the molecules of the solute on one side of the channel to diffuse to the other side. From $D = \frac{v_0^2}{t_0}$, this time can be estimated as $t_{mix} \sim \frac{w^2}{D}$ and the mixing distance is $d_{mix} \sim \frac{v_0 w^2}{D} = \nu P\dot{e}$. For usual values of the speed $v_0 = 100 \mu m s^{-1}$, lateral dimension of the channel $w = 100 \mu m$ and diffusivity $D \sim 5 \times 10^{-10} m^2 s^{-1}$, this distance turns as $d_{mix} \sim 1 \, \text{cm}$, which can be fit in a microfluidic device. Mixing of two streams along a channel is a very common situation, which actually arose in the design of the device for our work on the entrainment of genetic oscillators (Mondragón-Palomino et al., 2011). Because the mixing distance along a straight micro-channel is proportional to the Péclet number, $d_{mix}$ can be prohibitively long if, for example, the solute is a protein ($D \sim 10^{-6}$) or the velocity of the flow is faster. To address this issue, mechanical engineers have figured ways to enhance diffusion through the repetitive folding of fluid streams (Stroock et al., 2002; Kim et al., 2004; Kang et al., 2008).

**Recent applications of microfluidics in quantitative biology**

In the second part of this chapter we will review recent work that is exemplary of the power of microfluidics for the quantitative understanding and engineering of biology. First, we will discuss the work where microfluidics has brought new qualitative insights from single-cell studies, that cannot be obtained from coarse population-wide experiments. Then, we will look at research where microfluidic devices helped studying phenomena that involve large bacterial communities and communication between their members. The work reviewed here is mostly related to the study of microorganisms, in agreement with my personal interest in the area. However, there are many similar
examples of the impact of microfluidics on eukaryotic systems research which I will not discuss here.

**Microfluidics for single-cell microbiology**

Here we go over two recent works in which microfluidic devices were used to address, with single-cell resolution, the hypothesis of aging in symmetrically dividing bacteria (Wang et al., 2010) and the sources of phenotypical heterogeneity in the bacterium *Mycobacterium smegmatis*, which is a close relative of the pathogen *Mycobacterium tuberculosis*.

There is wide support for the idea that aging in unicellular organisms requires asymmetric cell division, as in the yeast *Saccharomyces cerevisiae* and the bacterium *Caulobacter crescentus*, in which cells produce a rejuvenated offspring (Nystrom, 2007; Ackermann et al., 2003). Nevertheless, recent work in the symmetrically dividing bacterium *Escherichia coli* challenged this concept (Stewart et al., 2005). *E. coli* bacteria grow by adding peptidoglycan to the lateral wall of the cell and then dividing at the center (Scheffers and Pinho, 2005). Upon cell division, two sister cells arise. Each sister gets a new pole and an old pole (Fig. 2.1). The age of an *E. coli* cell is defined in (Stewart et al., 2005) by the number of divisions (generations) since the cell has its oldest pole. Stewart et al. found that cells with older poles have a reduced growth rate, decreased offspring production and an increased death rate. These findings led authors to conclude that the sister cell that keeps the old pole should be considered as a mother cell that undergoes aging and gives rise to a rejuvenated offspring. Indeed, the authors argue that the inheritance of an old pole is the mechanism that generates asymmetry between the sister cells, making a mother and a daughter. The single-cell measurements were obtained from 94 two-dimensional colonies that grew from the same number of single cells for 8 cell divisions and on a solid surface of LB medium-agarose.

A recent study revisited the results of Stewart et al. (Wang et al., 2010). In this work the growth rate was measured on thousands of bacterial colonies that
Figure 2.1: Every time *E. coli* divides two sister cells arise. Each sister gets a new pole (cyan) and an old pole (red). The age of a symmetrically dividing *E. coli* cell is counted in the number of divisions (generations) the cell has had its oldest pole. Image modified from (Stewart et al., 2005) with permission from Public Library of Science.
are one cell wide. One such colony is obtained by growing a cell inside a rectangular microfluidic chamber that matches its width. The closed end of a chamber is occupied by the cell that keeps the old pole, and which can be observed for its lifespan. The open end of traps gives into a fluidic channel where the flow of media washes the offspring of the mother cell. A trap can host up to 8 cells at a time. Unlike the previous study in agar pads, this work found the growth and protein synthesis rates of mother cells is stable throughout cell’s lifespan (up to 250 generations).

\[\text{Figure 2.2: An } E. \text{ coli cell grows in the back of a microcavity for its lifespan. The chamber is } 25 \times 1 \times 1 \ \mu \text{m}^3 \text{ and only allows the tracking of one cell lineage because the progeny of the cell in the end of the trap is rapidly washed out by the flow of media. With this device it was found that the growth-rate of } E. \text{ coli is independent of the replicative age. Image reproduced from (Wang et al., 2010) with permission of Cell Press.}\]
The long single-cell trajectories allowed the authors to analyze the dynamics of cell growth and death beyond the capacity of any previous study. For example, they found the growth rate displays fast fluctuations with a time-scale of less than a generation, meaning that cells do not keep a memory of their growth rate beyond less than one cell division. On the other hand they were able to observe that the rate of filamentation of the mother cell, which is a phenotype closely associated to the SOS response, increases sharply after 50 generations. Their analysis showed that the time between filamentation events as a function of replicative age is distributed according to a power-law with a long tail, which reveals a long-term correlation or memory. The authors compared the death rate as a function of replicative age in wild type cells and mutants lacking the \textit{lexA} gene, which is involved in the SOS response, finding that that the death rate of the mutant population was constant while the death rate of wild type cells increased with their replicative age. The authors interpreted this as a suggestion that cell damage or a killing agent progressively accumulates and increases the probability of cell death but does not diminish the ability of cells to grow and divide. In other words, cell division and cellular death are decoupled in \textit{E. coli}.

This work is a prime example of the advantages of microfluidics. In the study by Stewart \textit{et al.} it is claimed cells were held in a constant concentration of nutrients and oxygen for the length of the experiment. While this may be true, cellular waste and other small molecules may accumulate in the two dimensional colonies, possibly having a detrimental effect on the growth rate of cells. The microfluidic design by Wang \textit{et al.} removed this obstacle.

In the case of the budding Yeast \textit{Saccharomyces cerevisiae}, the number of mitotic divisions a cell undergoes before dying (or replicative life-span) are counted by manually removing the daughter cells after they split from their mother using a micro-needle. This assay was introduced by Mortimer and Johnston 53 years ago and it stands as the best method, in spite of efforts to automate it (Mortimer and Johnston, 1959; Jarolim et al., 2004). This direct method is incompatible with the obtention of abundant data. However, some microfluidic designs have been put forward to automate single
cell measurements in the budding Yeast (Rowat et al., 2009). Rowat’s design is based on constraining the growth of a Yeast cell in a linear chamber. However, in this case the linear chamber is long to keep all the progeny of the single cell at the back of the chamber. In this way it is possible to reconstruct a genealogy starting from the first cell. This device cannot be used in aging experiments because the fluidic channel onto which the cell chamber opens is only 5 microns in height, and is easily obstructed by mother-daughter couples coming out of the trap. This device sets the stage for the development of improved designs where different measures of aging in yeast can be obtained simultaneously and we are confident they they will be available soon.

Clearly, retaining a single cell is advantageous for studying aging, but in many other instances it is useful to observe the growth of entire populations. Clonal cellular populations are usually a mix of phenotypes and such heterogeneous composition is driven by stochastic and deterministic mechanisms. It is widely accepted that a population is less susceptible to succumb when its variable composition is translated into a differentiated sensitivity to external stresses. However, no experimental proof for this hypothesis had been provided until recently. Aldridge and collaborators investigated the hypothesis that the variable outcomes upon antibiotic treatment of tuberculosis could be related to multiple phenotypes of the causative bacterium *Mycobacterium tuberculosis* (Aldridge et al., 2012).

To verify this idea, the division of the related, non-pathogenic, bacterium *Mycobacterium smegmatis* was observed for up to five generations. Two-dimensional micro-colonies were cultured from single cells within long, rectangular microfluidic chambers which are open on their shorter sides to micro-channels where media flows (Fig. 2.3 A). This simple microfluidic setup was enough to image multiple colonies for up to 5 generations, which span approximately 13 h. The authors found that, upon cell division, sister cells have different growth properties. The cell that inherits the mothers growth pole tends to elongate faster, while the other cell must regenerate a new growth pole and tends to elongate more slowly. As a result, the mycobacterial growth strategy generates a population with diverse growth rates and sizes. These two types of cells
were identified and termed as “alternators” and “accelerators” (Fig. 2.3 B). Finally, the authors found that the alternator and accelerator cells responded differently to antibiotic treatment. The ability of following multiple colonies with single cell resolution enabled the researchers to grow micro-colonies and know exactly what cells belong to each kind before stressing them with antibiotics. Access to abundant data allowed the authors to demonstrate their conclusions based on significant statistics.

**Bacterial group behavior**

There are numerous instances in which the cells in a colony or a tissue act in concert (Dunny and Leonard, 1997; Trosko et al., 1998; Waters and Bassler, 2005). Bacterial phenotypes such as pathogenicity or luminescence are supported by the ability of individual cells of communicating with their peers (De Kievit and Iglewski, 2000; Lupp et al., 2003). Studying the dynamics of such collaborative behaviors in the laboratory requires creating the conditions that are conductive to the formation of stable communities and to the exchange of information between cells.

Chemotaxis is the ability of bacteria to change the direction of swimming in response to a chemical gradient and this ability is used in some cases to search for food (Seymour et al., 2010). Bacteria can also secrete chemoattractants and chemorepellants, which as their name suggests, serve to attract and repel other cells. In the absence of flow, these two mechanisms may combine and produce a group of cells that moves coherently in search for food.

To study this phenomenon with single cell resolution, Saragosti et al. introduced thousands of cells into a straight microfluidic channel with rich media (Saragosti et al., 2011). Cells were centrifugated to one end of the channel and then they were allowed to swim freely. A wave of cellular concentration propagated along the channel as a result. The authors were able to measure the global features of the concentration front, while simultaneously tracking the trajectories of individual swimmers. These experiments were compared against the predictions of a model. Some parameters of the model were di-
Figure 2.3: A) Microfluidic setup for observing Mycobacterium cells and the pattern of deterministic variability in their lineage. B) Mycobacterium’s cell envelope grows at one pole and then divides. Each cell division gives rise to an alternator and an accelerator cell. The alternator is the cell that looses the growing pole and has to start a new one. The new pole grows on the opposite side of the lost growth pole. The accelerator is the cell that keeps the growing pole. Image reproduced with permission from the American Association for the Advancement of Science.
rectly obtained from the microscopic measurement (run speed, mean tumble frequency, modulation of tumble frequency as a function of chemoattractant and nutrients). The authors found that the only way to reproduce the velocity of the cell wave with their model was by integrating taking into account the observation that the orientational persistence of individual swimmers is modulated with direction.

Without the PDMS microfluidic channels, these experiments would not have been possible because the alternative chemotaxis assay (a capillary glass needle) does allow the observation of single-cell trajectories and the high concentration of cells used in this experiment may have exhausted the local supply of oxygen.

**Figure 2.4:** A) Space-time plot of the propagating bacterial concentration wave in a microfluidic channel. The inset shows the microscopic single-cell trajectories inside the wave. Individual trajectories were obtained by labeling some cells with GFP (red trajectory). B) The orientational persistence is a measure of how much a swimming cell changes its direction after a run, and it is measured by the SD of the distribution of the reorientation angle $\sqrt{<\alpha^2>}$. The orientational persistence has an orientational dependence and is the least in the direction of propagation of the wave (green quadrant). Image reproduced from (Saragosti et al., 2011) with permission of the Proceedings of the National Academy of Sciences.
Chapter 3

Entrainment of a Population of Synthetic Genetic Oscillators

Introduction

One focus of synthetic biology is the genome scale synthesis of DNA for the creation of novel cell types (Gibson et al., 2010). This approach could lead to cells with highly reduced genomic complexity, as genes that govern the ability to adapt to multiple environments are eliminated to construct specialized organisms for biotechnology and basic research. Another branch of synthetic biology involves the engineering of gene circuits, whereby mathematical tools are developed to systematically design and construct circuits from a standardized list of biological “parts” (Gardner et al., 2000; Elowitz and Leibler, 2000; Hasty et al., 2002; Basu et al., 2005; Endy, 2005; Stricker et al., 2008a; Tigges et al., 2009; Tamsir et al., 2010; Danino et al., 2010, PMID: 20090747). The engineering approach allows the construction of circuits that mimic natural networks to understand the design principles that underly a given network motif (Sprinzak and Elowitz, 2005; AU Mukherji and van Oudenaarden, 2009). In this context, molecular clocks are a natural application of synthetic biology, and recent efforts have led to a deeper understanding of the robustness and reliability of time keeping at the intracellu-
lar level (Elowitz and Leibler, 2000; Stricker et al., 2008a; Tigges et al., 2009; Danino et al., 2010).

Almost all organisms use molecular clocks to keep their physiology and behavior in synchrony with their surroundings (Bell-Pedersen et al., 2005). Such coordination is mediated by entrainment, whereby a population of intracellular clocks oscillate in unison guided by a common external signal (Johnson et al., 2003; Bell-Pedersen et al., 2005). Quantitative descriptions of entrainment that arise from the tight coupling of computational modeling and experimentation are challenging to develop because of the complexity of the underlying gene regulatory networks, in which dozens of genes are involved in the core clocks and hundreds more act as their modifiers (Zhang and Kay, 2010). Moreover, a quantitative description of inherently stochastic circadian clocks requires abundant long-term single-cell data which are technically challenging to obtain (Welsh et al., 2004; Yoo et al., 2004; Abraham et al., 2010). We combined synthetic biology, microfluidic technology (Whitesides et al., 2001), and computational modeling to investigate the fundamental process of entrainment at the genetic level.

The physics of entrainment and modeling results

The entrainment of any self-sustained oscillator can be characterized by comparing its natural period \(T_n\) and phase \(\phi\) to those of the external signal. When the period of the forcing signal \(T_f\) is sufficiently close to the natural period of the oscillator, the oscillator can be entrained. In the entrainment regime, the period of the oscillator \(T\) is equal to the forcing period \(T_f\), and the phase difference \(\Delta\phi\) between the oscillator and the forcing signal is fixed. In the plane defined by the period and strength of the external signal \((T_f, A)\) a triangular region near \(T_f/T_n = 1\) indicates where the oscillator is entrained (Fig. 3.1E and Appendix for Chapter 3). Entrainment may also occur near other rational values of \(T_f/T_n\). Collectively, these regions are known as Arnold tongues. The order of locking in each region is indicated by the ratio \(n : m\), which denotes that \(m\) oscillations of the clock correspond to \(n\) oscillations of the arabinose signal. We com-
puted the tongues for entrainment of order 1:1 and 2:1 with a deterministic model of the synthetic oscillator (Stricker et al., 2008a), in which we periodically modulated the arabinose concentration.

**Experimental results**

We used a synthetic oscillator that has coupled positive and negative feedback loops that are characteristic of many circadian gene-regulatory networks (Stricker et al., 2008a) (Fig. 3.1A). The green fluorescent protein (GFP) was used as a readout of the transcriptional activation state of the promoter that drives the expression of the oscillator genes. We stimulated the expression of the oscillator genes (araC and lacI) by periodically modulating the concentration of the transcriptional inducer arabinose, which acts on the positive feedback loop. Such stimulation is referred to as the forcing of the oscillator. To generate long-term single-cell data for comparison with computational modeling, we constructed microfluidic devices in which bacterial colonies can grow exponentially for at least 150 generations (Fig. 7.1). For each experimental run, we tracked the phase of the oscillations with respect to the arabinose signal in approximately 1600 cells (Fig. 3.1B, movies S1 and S6). The period of oscillations $T$ was measured as the peak-to-peak interval in the GFP fluorescence time series. The phase difference between an oscillator and the arabinose signal was calculated as $\Delta \phi = 2\pi \Delta T / T_f$, where $T_f$ is the period of the signal and $\Delta T$ is the measured time interval between a crest of arabinose and the immediate following peak of GFP fluorescence (Fig. 3.1C). Entrainment of the intracellular oscillations to the chemical signal was readily identified from color density maps of the fluorescence trajectories (Fig. 3.1D); by taking crests of GFP fluorescence as a marker of the phase, one can see that while in the autonomous set single cells are not always in phase with respect to each other, maxima in the forced colony occur almost simultaneously during most of the run.

To experimentally map the entrainment regions we first determined the natural period of the oscillator by tracking the expression of GFP of cells at constant inducer
Figure 3.1: We use single-cell data from time-lapse fluorescence experiments to investigate the entrainment of a synthetic oscillator. **A**, Architectures of eukaryotic circadian clocks and bacterial synthetic oscillators contain positive and negative feedback loops that are sensitive to external stimuli. **B**, Fluorescence images from a time-lapse experiment show coherent GFP oscillations (green) in a colony of single-cell oscillators subject to a 30 minute cycle of arabinose (red) (Supplementary Movie 1). **C**, Fluorescence time series of a single-cell oscillator (green). The concentration of arabinose (red) changes sinusoidally according to \([\text{ara}(t)] = 0.3 + A \sin (2\pi t/T_f)\) (% w/v), with \(A = 0.15\%\) and \(T_f = 30\) min. The intensity plot above the graph corresponds to the cell trace. **D**, Fluorescence intensity plots of free-running and forced oscillators. Each row in the two panels represents a single-cell trace. The top row of the forced set represents the modulated concentration of arabinose \((A = 0.15\%)\). **E**, Entrainment regions indicate which forcing periods \((T_f)\) and amplitudes \((A)\) result in locking of the oscillator according to a deterministic model (Appendix for Chapter 3). Entrainment of order 2:1 means that one oscillation peak is observed for two peaks of arabinose. \(T_n\) is the natural period of the oscillator. Images and cell traces shown in (B), (C) and (D, forced) correspond to point 4. Points 1-3 signal some parameter values explored experimentally.
**Figure 3.2:** Probability distributions of the relative phase of oscillators with respect to the external signal allowed the detection of entrainment. **A.** Probability distribution of the relative phase of free-running oscillators in several colonies with respect to a virtual sinusoidal signal of period $T_f = 30$ min. Constant concentrations of inducers were used, $[IPTG] = 2$ mM, $[ara] = 0.3$ %. **B.** Probability distributions of the relative phase for multiple forcing periods with amplitude $A = 0.075$ % (w/v). In the presence of the external stimulus, distributions acquired a preferred phase that depends on the forcing period $T_f$. **C.** As in (B) for stronger forcing with $A = 0.15$ %. Increased amplitude sharpens the peaks of the relative phase distributions with respect to those for $A = 0.075$ % as in $T_f = 15, 30,$ and 45 min. **D.** Intensity of entrainment as a function of the forcing period for the two values of the forcing amplitude. In each curve, two peaks centered near $T_f \sim T_n = 31.8$ min and $T_f \sim T_n/2 = 15.9$ min reveal the intervals of $T_f$ where the phase is locked to the arabinose input. For free-running oscillators $\rho$ is nearly zero (open circle). **E.** Relative phase as a function of time for three experiments from (B). Colored regions correspond to $\pm$ SD around the mean phase drift. Dashed lines indicate representative single cell traces.
concentrations (Supplementary Movie 2). Similar to its naturally occurring counterparts (Welsh et al., 2004; Yoo et al., 2004; Carr and Whitmore, 2005), the synthetic oscillator shows considerable fluctuations (Fig. 3.3A). Because the oscillators are not synchronized with respect to each other, their phases are uniformly distributed between $0$ and $2\pi$ (Fig. 3.2A). Given the natural period of approximately 32 minutes, we varied the period of the arabinose concentration from 6 to 60 minutes for two values of the amplitude. Coherent oscillations emerged over a range of periods that bounded the natural period (Supplementary Movie 3). Phase locking was characterized by a narrow peak in the phase distribution (Tass et al., 1998), which became difficult to discern as the period of the signal diverged from the natural period, but reappeared as the forcing period approached half of the natural period (Fig. 3.2B, Supplementary Movie 4). An increase in the forcing amplitude by a factor of two led to sharper distributions of the relative phase (Fig. 3.2C, Movies S2 and S5). To quantify the degree of phase locking, we used an entropy-based index ($\rho$) to characterize the width of the distributions (Tass et al., 1998); wider distributions imply less phase locking and lead to smaller values of $\rho$ (Appendix for Chapter 3). Accordingly, maxima of the entrainment index appeared at both the natural period and half the natural period (Fig. 3.2D).

The flattening of phase distributions and the decay of the phase-locking index around $T_f/T_n = 1/2$, 1 indicates the breaking of entrainment. To investigate this transition in more detail we examined the dynamics of the oscillation phase relative to the forcing signal in single cells. We chose three values of the forcing period that cross the left boundary of the computed main Arnold tongue (Figs. 3.1E and 3.2B). We used peak positions to determine the phases of the arabinose signal $\phi_{ara}(t)$ and of single cell oscillations $\phi_c(t)$, and calculated their difference $\Delta\phi(t) = \phi_{ara}(t) - \phi_c(t)$ (Fig. 3.2E). At the center of the entrainment region ($T_f = 33$ min), $\Delta\phi$ for most oscillators was nearly constant (Fig. 3.2E, blue envelope and curves). Near the boundary of the tongue ($T_f = 30$ min) there is a slow mean phase drift with a broad distribution (Fig. 3.2E, red envelope and curves); some cells exhibit phase drift (with an evidence of occasional phase slips) while other cells are still phase-locked. Finally, between the two Arnold tongues ($T_f =$
24 min), the rate of phase drift was even faster and almost uniform because the phases of most oscillators did not lock to the arabinose signal (Fig. 3.2E, green envelope and curves). The continuous phase drift indicates quasiperiodic behavior outside entrainment regions observed in the computation of Arnold tongues (Fig. 3.1E and Appendix for Chapter 3).

Figure 3.3: Probability distributions of the period of oscillations allowed us to find the forcing periods where the synthetic oscillator is frequency locked. A. Probability distribution for the period of free running oscillators in constant concentrations of inducers, $[\text{IPTG}] = 2 \text{ mM}$, $[\text{ara}] = 0.3 \%$. In forcing experiments, the concentration of arabinose oscillates sinusoidally around $[\text{ara}] = 0.3 \%$. We defined the natural period as the mean period of free oscillations, $T_n = 31.8 \text{ min}$ with standard deviation $\delta T = 5.7 \text{ min}$. B. Probability distributions of the period for multiple values of the forcing period ($T_f$) with $A = 0.075 \%$. For $T_f$ near $T_n = 31.8 \text{ min}$ or $T_n/2 = 15.9 \text{ min}$, the dispersion of the period is the least. C, Probability distributions of the period for multiple values of the forcing period with $A = 0.15 \%$. Period distributions for this higher amplitude can contain two modes (blue and brown). D, The ratio $T/T_f$ as a function of $T_f$ for the two forcing amplitudes, where $T$ is the mode(s) of the period distributions. The intervals of the forcing period where $T/T_f \sim 1:2$ provided evidence for entrainment of order 1:1 and 2:1 respectively.

We also used period distributions to characterize the response of the oscillator (Fig. 3.3). Forcing periods close to both the natural period and half the natural period reduce the spread of the period distribution in a manner similar to that observed with light pulses entraining peripheral clocks (Carr and Whitmore, 2005). For a lower
amplitude of the arabinose signal, oscillators were entrained over an interval of periods that was consistent with the width of the 1:1 phase locking regime determined using the entropy-based measure (Figs. 3.3D and 3.2D). For a larger forcing amplitude, the 1:1 plateau extended over a larger interval of periods, and a 2:1 plateau indicated the presence of the higher order resonance. Some of these distributions displayed two modes, which presumably indicated simultaneous occurrence of 1:1 and 2:1 frequency locking.

**Discussion and modelling**

Direct comparison between our experimental results and the computed Arnold tongues indicated that the locations of the experimental entrainment plateaux correspond closely to the regions where frequency locking is predicted (Fig. 3.4A). The width of the plateau increased with the amplitude of the forcing signal, as follows from classical theory (Pikovsky et al., 2001). However, the experimental entrainment regions were consistently wider than the computed Arnold tongues. The major discrepancy between the naive model and experiment is that the model assumes that all oscillators are identical and have the same natural period, whereas the bacterial colony exhibits a broad distribution of periods (Fig. 3.3A).

The observed variability of the oscillatory dynamics can be attributed to both intrinsic and extrinsic origins (Swain et al., 2002). We incorporated both sources of variability into our model because it is difficult to ascertain *a priori* which one dominates. We used a Gillespie algorithm (GILLESPIE, 1977) to simulate the stochastic model of the oscillator network with intrinsic noise only (the kinetic parameters of all oscillators were set to be identical). Although the simulated distributions appeared similar to experimental data (Figs. 7.1-7.4), the stochastic model did not account for the higher order (2:1) resonance entrainment, the period bimodality, or the wider entrainment regions (Figs. 7.1-7.4 and 3.4B-3.4C black circles). We therefore modeled extrinsic variability by varying the kinetic parameters of the deterministic model across a population of 550 cells. In particular, we assumed that the rates of transcription, trans-
lation, enzymatic degradation by proteases, and plasmid copy numbers were normally
distributed around their nominal values. Using a coefficient of variation (CV) of 0.15,
close to CV=0.18 of the experimental probability distribution of the free-running pe-

diod, we obtained good agreement between the modes of simulated and experimental
period distributions (Fig. 3.4D black circles and Figs. 7.6 and 7.8). Accordingly, the
distributions of the relative phase and the peaks in the curves for the intensity of phase
locking were comparable (Fig. 3.4E and Figs. 7.5 and 7.7). Deterministic simulations
with randomized parameters accounted for the width of both the 1:1 and 2:1 entrainment
regions. Simulations also reproduced peaks in bimodal period distributions (Figs. 7.6
and 7.8).

These results can be readily understood in the context of the phase dynamics.
For fixed concentrations of arabinose and IPTG (isopropyl-β-D-thiogalactopyranoside),
the natural period of the oscillator $T_n$ is a function of the parameters of the model, for
instance the rates of transcription, translation, enzymatic degradation, and of the ratio
of activator to repressor plasmids. Therefore, variability in these parameters will lead
to the observed variability in the periodicity of free-running oscillations (Fig. 3.3A).
Each individual oscillator will respond differently to the forcing arabinose signal, and
depending on its natural frequency it may or may not entrain. If the natural frequency
distribution occupies an interval of given width, the entrainment interval will broaden
by the same amount (Appendix for Chapter 3). Moreover, the broad distribution of nat-
ural frequencies of oscillators explains the occurrence of bimodal period distributions.
Indeed, if the forcing frequency is shifted with respect to the peak of the free-running
frequency distribution, both the entrainment peak at $T_f$ and the “free” peak at $T_n$ may
coexist.

Because circadian oscillators can be entrained by stimuli that act on different
components (Boothroyd et al., 2007), we explored the entrainment of the oscillator
through the periodic modulation of the concentration of IPTG (Fig. 3.1A). We did this
through deterministic simulations of the model, in which arabinose was kept constant
and the concentration of IPTG oscillated sinusoidally (Appendix for Chapter 3). We
Figure 3.4: Computational modeling shows that extrinsic sources are the dominant contribution to variability. Blue and red data points indicate experimental data for $A = 0.075 \%$ and $A = 0.15 \%$, respectively. Error bars represent $\pm$ SD. A, Experimental values of $T/T_f$ alongside computed entrainment regions (purple), which are shifted with respect to each other to account for the gap between the $T/T_f = 1$ and $T/T_f = 2$. Entrainment was observed for $T_f$ outside the computed entrainment areas. B, D, Same as in (A) along with the prediction for the ratio $T/T_f$ (open circles) from a stochastic model (B), and from a deterministic model with distributed parameters in a set of 550 oscillators (D). Unlike the oscillator subject to intrinsic noise (B), the oscillator with distributed kinetic parameters became phase locked outside computed entrainment regions (D). The ratio $T/T_f$ diverges from 1 or 2 outside Arnold tongues (B). C, E, Experimental values of the intensity of entrainment $\rho$ alongside the prediction (black circles) from a stochastic model (C) and a deterministic model with distributed parameters in a set of 550 oscillators (E). Intrinsic variability destroys the resonance around $T_f/T_n = 0.5$ (C), while the model with distributed parameters captures it (E). F, Main entrainment region for forcing with a sinusoidal IPTG signal of amplitude $A_{IPTG}$ for three concentrations of arabinose from a deterministic model (Appendix for Chapter 3). When the oscillator is forced through its negative feedback loop (Fig. 3.1A), the range of entraining frequencies increases with the constant arabinose concentration (strength of positive feedback loop).
found a similar behavior to forcing with arabinose, with a main entrainment region that widened with the amplitude of change in IPTG concentration. Because the concentration of arabinose defines the strength of the positive feedback through the AraC-DNA binding rate, we used different values to explore how entrainment depends on the strength of positive feedback. Lower concentrations of arabinose yielded narrower Arnold tongues (Fig. 3.4F). In other words, a weaker positive loop makes the oscillator less entrainable.

**Conclusions and future work**

We have shown how the coupling of synthetic biology, microfluidic technology, and computational modeling can be used to explore the complex process of entraining molecular clocks. Our results indicated that the positive feedback loop widens the entrainment region for single cells, providing insight into the possible role of positive feedback in the robust adaptation of variable clocks to complex environments (Rand et al., 2004). The observation of higher order entrainment and the wider entrainment regions allowed us to discriminate intrinsic sources in favor of extrinsic noise as the main contribution to stochastic variability in computational modeling of the clock. Other manifestations of strong cell-cell variability in gene networks have been quantified (Tu and Grinstein, 2005; Fischer-Friedrich et al., 2010). Although cell-cell variability may be deleterious to biological function, variable entrainment properties across a population may provide increased flexibility to the various signals that reset clocks. This may be relevant in the context of multicellular circadian systems where uncoupled peripheral oscillators display variability and are exposed to multiple signals (Welsh et al., 2004; Yoo et al., 2004; Carr and Whitmore, 2005; Dibner et al., 2010). Other properties at the cell and tissue level have been found to contribute to the flexibility of circadian clocks; recent work found an effect of the strength of coupling between cell clocks on the range of entrainment in mammalian circadian clocks (Abraham et al., 2010).
Acknowledgements

This work was supported by the National Institutes of Health and General Medicine (grant R01GM69811), the San Diego Center for Systems Biology (grant P50GM085764), and CONACyT (Mexico, grant 184646 to O.M.-P.). Single-cell data obtained from automated tracking and lineage reconstruction is available online at http://biodynamics.ucsd.edu/downloads.

Chapter 4

A Synchronized Quorum of Genetic Clocks

Introduction

The engineering of genetic circuits with predictive functionality in living cells represents a defining focus of the expanding field of synthetic biology. This focus was elegantly set in motion ten years ago with the design and construction of a genetic toggle switch and oscillator, with subsequent highlights that have included circuits that are capable of generating patterns, shaping intracellular noise, detecting edges in an image, and counting discrete events. Here, we describe an engineered gene network with global intercellular coupling that is capable of generating synchronized oscillations in a growing population of cells. Using microfluidic devices tailored for cellular populations at differing length scales, we investigate the collective synchronization properties along with spatiotemporal waves occurring on millimeter scales. We use computational modeling to quantitatively describe the observed dependence of the period of bulk oscillations on the flow rate and oscillatory amplitude. The synchronized genetic clock sets the stage for the use of microbes in the creation of a macroscopic biosensor with an oscillatory output. In addition, it provides a specific model system for the generation of
a mechanistic description of emergent coordinated behavior at the colony level.

Centralized clocks are of fundamental importance in the coordination of rhythmic behavior among individual elements in a community or a large complex system. In physics and engineering, the Huygens paradigm of coupled pendulum clocks (Mirollo and Strogatz, 1990; Pikovsky et al., 2002; Bennett et al., 2002) has permeated diverse areas from the development of arrays of lasers (Vladimirov et al., 2003) and superconducting junctions (Wiesenfeld et al., 1996) to GPS (Lewandowski et al., 1999) and distributed sensor networks (Li et al., 2002). Perhaps one of the most bizarre (and unintended) examples of synchronization in engineering involved London’s Millennium Bridge (GC, 2005), which originally had a resonant frequency that was near the walking frequency of a typical pedestrian. On opening day, out of step pedestrians set the suspension bridge to wobble with a motion that coupled back on the pedestrians and induced synchronized marching which, in turn, further amplified the swaying of the bridge.

In biology, synchronized rhythms are abound, with behavioral examples that include flashing fireflies (Buck and Buck, 1968), swarming locusts (Buhl et al., 2006), and activity waves in ant colonies (Boi et al., 1999). In terms of human physiology, a vast range of intercellular coupling mechanisms lead to synchronized oscillators which govern fundamental processes such as somitogenesis, cardiac function, respiration, insulin secretion, and circadian rhythms (Winfree, 1967; Mirollo and Strogatz, 1990; Elson et al., 1998; Jiang et al., 2000; Glass, 2001; Young and Kay, 2001; Chabot et al., 2007; Kerckhoffs et al., 2009). Typically, synchronization helps stabilize a desired behavior arising from a network of intrinsically noisy and unreliable elements. Sometimes, however, the synchronization of oscillations can lead to a severe malfunction of a biological system, as in epileptic seizures (Grenier et al., 2003).

There is widespread interest in the use of synthetic biology to recreate complex cellular behavior from the underlying biochemical reactions that govern gene regulation and signaling. Synthetic biology can be broadly parsed into efforts aimed at the large-scale synthesis of DNA and the forward engineering of genetic circuits from
known biological components. In the area of DNA synthesis, pathways have been perturbed and replaced (Isalan et al., 2008) in an effort to understand the network motifs and transcriptional regulatory mechanisms that control cellular processes and elicit phenotypic responses (Alon, 2007b). On a larger scale, progress has been made towards the creation of entire genomes, providing new insights into what constitutes the minimal set of genes required for microbial life (Gibson et al., 2008). The genetic circuits approach (Hasty et al., 2002; Endy, 2005) involves the use of computational modeling in the design of relatively small genetic circuits. Here, the original toggle switch (Gardner et al., 2000) and oscillator (Elowitz and Leibler, 2000) have inspired the design and construction of circuits capable of controlling cellular population growth (You et al., 2004), generating patterns (Basu et al., 2005), triggering biofilm development (Kobayashi et al., 2004), shaping intracellular noise (Austin et al., 2006), detecting edges in an image (Tabor et al., 2009), and counting discrete cellular events (Friedland et al., 2009). In the context of rhythmic behavior, there have been recent successes in the construction of intracellular oscillators that mimic naturally occurring clocks (Atkinson et al., 2003; Stricker et al., 2008a; Tigges et al., 2009; Fung et al., 2005). Theoretical work has shown how the introduction of an autoinducer in oscillator designs can potentially lead to synchronized oscillations over a population of cells (McMillen et al., 2002; Garcia-Ojalvo et al., 2004).

**Synchronized genetic oscillators**

The synchronized oscillator design (Fig. 4.1a) is based on elements of the quorum sensing machineries in *Vibrio fisheri* and *Bacillus Thuringiensis*. We placed the *luxI* (from *V. fisheri*), *aiiA* (from *B. Thuringiensis*) and *yemGFP* genes under the control of three identical copies of the *luxI* promoter. The LuxI synthase enzymatically produces an acyl-homoserine lactone (AHL), which is a small molecule that can diffuse between cells and mediates intercellular coupling. It binds intracellularly to the constitutively produced LuxR, and the LuxR-AHL complex is a transcriptional activator for
the *luxI* promoter (Waters and Bassler, 2005). AiiA negatively regulates the promoter through the effective degradation of AHL by catalyzing the degradation AHL (Liu et al., 2008). This network architecture, whereby an activator activates its own protease or repressor, is similar to the motif used in other synthetic oscillator designs (Atkinson et al., 2003; Stricker et al., 2008a; Tigges et al., 2009) and forms the core regulatory module for many circadian clock networks (Glossop et al., 1999; Young and Kay, 2001; Lakin-Thomas and Brody, 2004).

![Diagram](image)

**Figure 4.1:** Synchronized genetic clocks. (a) Network Diagram. The luxI promoter drives production of the *luxI*, *aiiA*, and *yemGFP* genes in three identical transcriptional modules. LuxI enzymatically produces a small molecule AHL, which can diffuse outside of the cell membrane and into neighboring cells, activating the *luxI* promoter. AiiA negatively regulates the circuit by acting as an effective protease for AHL. (b) Microfluidic device used for maintaining *E. coli* at a constant density. The main channel supplies media to cells in the trapping chamber, and the flow rate can be externally controlled in order to change the effective degradation rate of AHL. (c) Bulk fluorescence as a function of time for a typical experiment in the microfluidic device. The red circles correspond to the image slices in (d). (d) Fluorescence slices of a typical experimental run demonstrate synchronization of oscillations in a population of *E. coli* residing in the microfluidic device (Supplementary Movie 1). Inset in the first snapshot is a 100x zoom of cells.

Most quorum sensing systems require a critical cell density for generation of coordinated behavior (Reading and Sperandio, 2006). To control cell density, we moni-
tored the synchronized oscillator cells (denoted TDQS1) at the single cell level by time-
lapse fluorescence microscopy using microfluidic devices (Cookson et al., 2005). These
deVICES consist of a main nutrient-delivery channel that feeds a rectangular trapping
chamber (Fig. 4.1b). Once seeded, a monolayer of *E. coli* cells grow in the chamber
and are eventually pushed into the channel where they then flow to the waste port. This
device allows for a constant supply of nutrients or inducers and the maintenance of an
exponentially growing colony of cells for more than four days. We found that chamber
sizes of 100 x (80-100) µm were ideal for monitoring the intercellular oscillator, as they
allowed for sufficient nutrient distribution and optimal cell and AHL densities. In the
context of the design parameters, the flow rate can be modulated in order to change the
local concentration of AHL. In addition, the device can be scaled up in order to permit
the observation of spatial waves over longer length scales (see below).

After an initial transient period, the TDQS1 cells exhibit stable synchronized
oscillations which are easily discernible at the colony level (Figs. 4.1c,d, and Supple-
mentary Movies 1-3). The dynamics of the oscillations can be understood as follows.
Since AHL diffuses downstream and is degraded by AiiA internally, a small colony
of individual cells cannot produce enough inducer to activate expression from the *luxI*
promoter. However, once the population reaches a critical density, there is a “burst”
of transcription of the *luxI* promoters, resulting in increased levels of LuxI, AiiA, and
GFP. As AiiA accumulates, it begins to degrade AHL, and after a sufficient time, the
promoters return to their inactivated state. The production of AiiA is then attenuated,
which permits another round of AHL accumulation and another burst of the promoters.

In order to determine how the effective AHL diffusion rate affects the period
of the oscillations, we conducted a series of experiments at various channel flow rates.
At high flow rate, the oscillations stabilize after an initial transient and exhibit a mean
period of 90±6 minutes and mean amplitude of 54±6 GFP AU (Fig. 4.2a, Supple-
mentary Movie 3). At low flow rate, we observed a period of 55±6 minutes and
an amplitude of 30±9 GFP AU. Interestingly, the waveforms were distinct, with the
slower oscillator reaching a trough near zero after activation and the faster oscilla-
tor decaying to levels above the original baseline (Fig. 4.2b). We swept the flow rate from 180-280 µm/min and observed an increasing oscillatory period from 55-90 minutes (Fig. 4.2c). In addition, we found the amplitude to be proportional to the period of the oscillations (Fig. 4.2d), which is consistent with the “degrade and fire” type oscillations (Mather et al., 2009) observed in a previously reported intracellular oscillator (Stricker et al., 2008a).

![Graphs](image_url)

**Figure 4.2:** Dynamics of the synchronized oscillator under multiple microfluidic flow conditions (Supplementary Movies 2 and 3). (a) At around 90 minutes, cells begin to oscillate synchronously after reaching a critical density in the trap. (b) The period and amplitude increase for higher flow rates. Magenta curve is at low velocity (240 µm/min), blue is at higher velocity (280 µm/min). (c) Period as a function of velocity in the main channel showing tunability of period between 55-90 minutes. (d) Period vs. amplitude for all experiments. Magenta circles (c,d) are data from 84 and 90 µm traps, blue crosses are 100 µm traps.

In experiments conducted at low flow rate, we observed the spatial propagation of the fluorescence signal across the 100 µm chamber. In order to investigate these spatiotemporal dynamics in more detail, we redesigned the microfluidic chip with an extended 2mm trapping chamber (Appendix for Chapter 4). Snapshots of a typical
experimental run are presented in Fig. 4.3a (Supplementary Movies 4 and 5). A few isolated colonies begin to grow and subsequently merge into a large monolayer that fills the chamber (Fig. 4.3a: 66 minutes). At 100 minutes, there is a localized burst of fluorescence that propagates to the left and right in subsequent frames (Fig. 4.3a: 100-118 minutes). A second burst occurs near the original location and begins to propagate to the left and right as before.

**Spatiotemporal Dynamics**

To illustrate the spatiotemporal information contained in an entire 460-minute image sequence, we plot the fluorescence intensity as a function of chamber distance and time (Fig. 4.3b). Note the correspondence of this space-time plot to the images in Fig. 4.3a. During the first 100 minutes, there is no activity and the space time plot is blue, indicating no fluorescence. Then at 100 minutes, there is an orange spot at around 1350 μm, corresponding to the burst in Fig. 4.3a. In the space-time plot, propagation of a wave to the left and right appears as an green-yellow line with positive concavity. The larger slope to the left of the burst-origin indicates that the leftward moving wave is traveling slower (≈25 μm/min) than the rightward wave (≈35 μm/min). Subsequent waves originating from a nearby location arise as additional orange-yellow intensity lines. The second and 3rd intensity lines indicates an “annihilation event”, where a leftward moving and rightward moving wave collide and annihilate each other. While these events are striking in the movies (Supplementary Movies 4 and 5), they appear subtly in the space-time plot at locations where positive and negative concavity meet(300-400 μm in 2nd intensity line and on). As the traveling wave gets further from a burst location it breaks off into a packet (170 minutes) which travels leftward at 12.5 μm/min initially, and slows to 8.5 μm/min towards the end of the trap where the cell density is lower (between 118-200 minutes). The corresponding cell-density space-time plot shows that a higher density of cells is first reached at the center of the colony and is minimal towards the left-moving edge (Fig. 4.3c and Supplementary Movie 4). As a result, the critical
cell and AHL densities for wave propagation are reached at different times and spatial locations.

We also investigated how the intercellular oscillator behaves in a three dimensional colony growing in a 400x1000x4.0 μm microfluidic chamber (Figs. 4.3d, 4.3e, and Supplementary Movie 6). In this device, the colony grows radially over the course of 180 minutes without fluorescing until it reaches a size of approximately 100 μm. At this time, a large fluorescence burst originates from the center of the colony, with a bright band near the center (Fig. 4.3d: 228 minutes). During this first burst (273 minutes), the bright band shows that cells at an intermediate cell density have a larger amplitude and longer period than cells near the front or in the interior. As the colony expands an additional 50-100 μm in diameter, a second burst of fluorescence occurs at a similar intermediate cell density. Subsequent oscillations are seen as the cell growth front propagates, while weak oscillations arise and quickly die inside the colony.

Quantitative modeling

In order to quantitatively describe the mechanisms driving bulk synchronization and wave propagation, we developed a computational model using delayed differential equations for protein and AHL concentrations (Appendix for Chapter 4). While conceptually the nature of oscillations is reminiscent of the degrade-and-fire oscillations observed in a dual delayed feedback circuit (Stricker et al., 2008a; Mather et al., 2009), an important difference is the coupling among genetic clocks in different cells through extracellular AHL. The modeling of this coupling, and the related cell density dependence, allowed us to explain most of the non-trivial phenomenology of the spatiotemporal quorum clock dynamics.

A broad range of model parameters lead to oscillations (Figs. 4.4a-d), though there is a distinct absence of oscillations at small and large cell densities for low to medium flow values (Fig. 4.4c). The qualitative nature of the oscillations can be explained using Fig. 4.4a. Each period begins with the latent accumulation of both AiiA
Figure 4.3: Spatiotemporal dynamics of the synchronized oscillators. (a) Snapshots of the GFP fluorescence superimposed over brightfield images of a densely packed monolayer of *E. coli* cells are shown at different times after loading (Supplementary Movies 4 and 5). Traveling waves emerge spontaneously in the middle of the colony and propagate outwards with the speed of $\sim 8-35 \mu m/min$. At later times waves partially lose coherence due to inhomogeneity in cell population and intrinsic instability of wave propagation (see Modeling Box). (b) Corresponding space-time diagram showing the fluorescence of cells along the center of the trap as a function of time. (c) Snapshots of the GFP fluorescence superimposed over the brightfield images of a three-dimensional growing colony of *E. coli* cells at different times after loading (Supplementary Movie 6). Bursts of fluorescence begin when the growing colony reaches a critical size of about $100 \mu m$. These bursts are primarily localized at the periphery of the growing colony. (d) Corresponding space-time diagram showing fluorescence of cells along a horizontal line through the center of the growing colony.
and LuxI, which after a delay, burst rapidly to high values. That burst suppresses AHL and further production of AiiA and LuxI which then decay enzymatically, after which the process repeats. As expected, the period of the oscillations is roughly proportional to the enzymatic protein decay time. The period grows with the external AHL flow rate (effective degradation) and the amplitude of the oscillations, in good agreement with the experiments (compare Fig. 4.4b with Figs. 4.3c and 4.3).

We modeled the collective spatiotemporal dynamics of the clocks by generalizing the bulk model to include the coupling of individual oscillators through extracellular AHL. The model consists of a one-dimensional array of “cells”, each of which is described by the same set of delay-differential equations coupled to a common, spatially nonuniform field of extracellular AHL. The latter is described by a linear diffusion equation with sources and sinks due to AHL diffusion through the cell membrane and dilution. A small AHL perturbation in the middle of the array, initiates waves of LuxI concentration (Fig. 4.4c), in excellent agreement with the experimental findings (compare Figs. 4.3b and 4.4c). The velocity of the front propagation depends on the external AHL diffusion coefficient $D_1$ (Fig. 4.4f and Appendix for Chapter 4), and for experimentally relevant values of $D_1$, the simulated front velocity is in good agreement with experimental data. In addition, cell density plays an important role in wave propagation. In order to model the evolution of the three dimensional colony (Figs. 4.3c and 4.3d), we set the functional form of the cell density to be an expanding “Mexican hat”, as observed in the experiments. Oscillations are then suppressed by the high density of cells in the middle of the colony, and LuxI bursts only occur on the periphery of the growing colony of cells. This phenomenology is also in excellent agreement with our experimental findings (compare Figs. 4.4d and 4.3d).

**Emergence**

On a fundamental level, the synchronized oscillations represent an emergent property of the colony that can be mechanistically explained in terms of the clock design.
Figure 4.4: Modeling of synchronized genetic clocks. (a) A typical time series of concentrations of LuxI (cyan circles), AiiA (blue circles), internal AHL (green line), and external AHL (red line). LuxI and AiiA closely track each other, and are anti-phase with the concentrations of external and internal AHL. (b) Period of oscillations as a function of the flow rate $\mu$ at cell density $d = 0.5$ (top panel). Period as a function of the amplitude of oscillations for the same cell density (bottom panel). (c) Period and amplitude as a function of cell density and AHL decay rate $\mu$. Oscillations occur over a finite range of cell densities, and period increases with $\mu$ after the bifurcation line is crossed. The results in (c) and (d) compare favorably with the experimental results in Figs. 4.2c and 4.2d. (d) Speed of wave front propagation as a function of the diffusion coefficient $D_1$. The numerical data scale as $V \sim D_1^{1/2}$ (red line). (e) Space-time diagram of traveling waves propagating through a uniform array of cells corresponding to the experiment depicted in Figs. 4.3a and 4.3b. (f) Space-time diagram of bursting oscillations in a growing cell population corresponding to the experiments in Figs. 4.3c and 4.3d.
Oscillations arise because the small molecule AHL plays a dual role, both enabling activation of the genes necessary for intracellular oscillations and mediating the coupling between cells. Since unbounded growth of the colony leads to an accumulation of AHL that ultimately quenches the bulk oscillations, we used open-flow microfluidic devices to allow for the flow of AHL away from the colony. At low cell densities, oscillations do not occur because intracellular gene activation is decreased as AHL diffuses across the cell membrane and out of the chamber. At intermediate cell densities (i.e., a full chamber), the increased production of AHL in each cell acts to mitigate the outward flow such that activation of the genes can occur in a rhythmic fashion, and colony-wide oscillations emerge in a seemingly spontaneous fashion.

A natural question arises regarding the behavior of individual cells in the absence of coupling. While experimentally we cannot turn off the coupling while maintaining intracellular gene activation, we addressed this question using simulations by artificially setting the AHL diffusion rate across the membrane to zero (with the other parameters fixed). We find that individual cells oscillate independently for any cell density since they are completely decoupled from the environment and each other. This result indicates that the coupling through AHL diffusion provides a means for the synchronization of individual oscillators at intermediate cellular concentrations.

**Perspective and outlook**

In the mid seventeenth century, Chirstiaan Huygens serendipitously observed that two pendulum clocks oscillated in synchrony when mounted to a common support beam (Bennett et al., 2002). While observations of synchronization in nature surely predate the age of enlightenment, Huygens is credited as the first to systematically characterize the synchronization of oscillators in terms of a known coupling mechanism (which, in the case of the pendulums, he deduced as vibrations in the common support). We have shown how quorum sensing can be used to couple genetic clocks, leading to synchronized oscillations at the colony level. Given the single-cell variability and
intrinsic stochasticity of most synthetic gene networks (Ozbudak et al., 2002; Elowitz et al., 2002; Atkinson et al., 2003; Stricker et al., 2008a; Austin et al., 2006), the use of quorum sensing is a promising approach to increasing the sensitivity and robustness of the dynamic response to external signals. Along these lines, our results set the stage for the design of networks that can function as spatially distributed sensors or synthetic machinery for coupling complex dynamical processes across a multicellular population.

Acknowledgements

We thank Matt Bennett, Kurt Wiesenfeld, and Jim Collins for stimulating discussions during the preparation of the manuscript. This work was supported by the National Institutes of Health and General Medicine (GM69811) and the DOE CSGF fellowship (TD).

Chapter 5

Streaming Instability in Growing Cell Populations

Introduction

Microorganisms employ a wide range of cooperative strategies for responding to adverse environmental conditions (Shapiro, 1998; Bassler, 2002; Stoodley et al., 2002; Donlan, 2002; Stewart and Franklin, 2008). In many cases, these strategies lead to intricate patterns and complex shapes in bacterial colonies (Ben-Jacob et al., 1995; Levine et al., 1996; Dockery and Klapper, 2002). While such patterning is usually associated with long-range cell signaling and motility (Park et al., 2003), microorganisms are often found in dense communities where direct cellular contact plays an important role in the dynamics of colony formation (Tolker-Nielsen et al., 2000; Drasdo, 2000; Goriely and Ben Amar, 2005). Here, we use microfluidic traps to characterize a general streaming instability occurring in a confined colony of non-motile bacteria. In order to investigate the mechanism driving the streaming instability, we develop a continuum model and complementary discrete-element simulations with cells modeled as growing and dividing soft spherocylinders which adapt their size and mobility to local chemical microenvironments. Given how these simulations readily generate a growing colony that
closely resembles the experimental patterning, we conclude that the streaming mechanism is related to the spatial heterogeneity of the colony due to chemical concentration gradients within the confined space.

**Experimental results**

In order to study bacterial colony growth in a confined environment, we constructed microfluidic devices featuring two types of traps (open and side) capable of sustaining a two-dimensional colony of non-motile bacteria *E. coli* over many generations. Open traps are typically $\sim 1 \mu m$-deep rectangular regions of different horizontal dimensions (up to $200 \times 200 \mu m^2$) embedded in the middle of the 6–10 $\mu m$-deep main channel (see Fig. 5.1 a,b). The external fluid flow through the main channel ($\sim 50 \mu m/sec$) delivers nutrients to the open boundaries of the trap, allowing for their diffusion into the interior of the trap. The fluid flow in the channel also removes metabolic waste and cells that escape from the trap. Side traps have similar dimensions but are embedded in the side walls of the main channel and have only one open boundary (Fig. 7.17).

Details of our experimental protocol are given in the Appendix for Chapter 5. In the beginning of each experiment we placed a few bacteria inside the trap and waited several hours (mean cell division time was about 20 min) until the colony grew and filled the trap region completely. The subsequent growth was balanced by a significant expansion flow towards the open boundaries of the trap. We found that the expansion flow of growing cells inside the traps was surprisingly non-uniform. Figure 5.1 c illustrates that cells escape into the main channel in narrow rapidly-moving *streams* (red) that bypass regions of almost stagnant cells (yellow) localized near the open boundaries (see also Supplementary Movie 1). This behavior was quantitatively analyzed by particle image velocimetry software MatPIV (Fig. 5.1 c,d). The cell streams are dynamic (see Fig. 5.1 d); the number and positions of streams fluctuates over the duration of a typical experiment. More detailed inspection of the cells inside the traps revealed that stagnant cells are generally thicker than rapidly moving cells comprising the streams. Furthermore,
Figure 5.1: A. Schematic view of the microfluidic device with a long open trap. Cells are seeded into a cell trap (white rectangle) situated in the middle of the main channel (in black); B. Zoomed view of a section of the cell chamber in A; D. Magnitude of the vertical component of cell velocity overlaid on a phase contrast image of the trap at time 210 min of the run shown in Movie S1; E. Space-time plot of the cells’ exit velocity component averaged over the lower 20 µm of the trap in C.
the cell size is generally dependent on its distance from the open boundary of the trap: in the nutrient-poor and waste-rich trap interior the cell diameter is only half of that near the open boundaries (where diameter is about 1µm), see Fig. 7.19.

The instability in cellular streams can be understood in terms of the interplay between the cell size and its mechanical properties. Since the trap height is nearly equal to the cell diameter, the mechanical interaction between the cells and the top and bottom walls of the trap affects their mobility. Under the same pressure gradient, smaller cells experience less friction and move faster, while larger cells experience higher friction and move slower. As cells move towards the open boundary, they grow larger in diameter, which leads to their reduced mobility. The streaming instability occurs when the growth rate of the diameter is comparable to the time a cell spends moving from the back of the trap to the open boundary. Under this condition, larger cells can effectively stop moving and form obstacles that permit streaming patterns to emerge for fast-moving cells (Supplementary Movie 1).

![Figure 5.2](image)

**Figure 5.2:** Stability analysis of the 2D continuum model, using \( c(z) = A + (z/L_z)^2 \), \( L_z = 1, \alpha = 1 \). a. Instability domain in the parameter plane \( (A, \gamma) \) for different values of viscosity coefficient \( \mu \). Streaming for a given \( \mu \) occurs for \( A \) below the corresponding curve; b. Velocity field for the eigenfunction near the onset of a streaming instability, with wave vector \( k = 2, \gamma = 1, A = 0.045, \mu = 0.001 \). Since the flow is inverted for \( \pi/4 \leq x \leq 3\pi/4 \), only half the \( x \)-period is displayed.
Continuum theory

In order to quantitatively investigate the mechanism of cell streaming during colony growth, we developed a continuum model of the colony dynamics which generalizes equations of two-dimensional incompressible fluid dynamics to include the effects of cell replication and drag force, the latter arising from the interaction of the cells with the floor and ceiling of the trap. We did not model the effects of cell shape and position-dependent growth rate here since they do not appear to be essential for the basic streaming mechanism.

We describe the incompressible “cell fluid” by the following equations:

\[ \frac{D\vec{v}}{Dt} = -\vec{\nabla}p - g(f)\vec{v} + \mu \nabla^2 \vec{v} \]  
(5.1)

\[ \frac{Df}{Dt} = \gamma (c(\vec{r}) - f) \]  
(5.2)

\[ \nabla \cdot \vec{v} = \alpha \]  
(5.3)

with \( \vec{v} \) the cell velocity field, \( \alpha \) the volumetric growth rate of the “cell fluid”, \( p \) the pressure field, \( f \) a field characterizing cell diameter, \( g(f) \) an \( f \)-dependent coefficient of (top and bottom) friction for cells moving in the shallow trap, \( \gamma \) the rate of \( f \) adaptation to the local chemical environment, \( \mu \) a coefficient of effective viscosity for cell flow, \( c(\vec{r}) \) the equilibrium value of \( f \) for a cell staying at point \( \vec{r} \) and \((D/Dt)\equiv\partial/\partial t+\vec{v} \cdot \vec{\nabla})\). \( c(\vec{r}) \) is chosen to be highest near an open boundary of the cell trap, such that cells become largest if stationary near the trap opening. The friction coefficient \( g(f) \) is assumed to nonlinearly increase with \( f \), due to the appearance of strong contact friction between the cell and the trap for large cells. In all results given below we have used \( g(f) = f^2 \), although the specific form of the nonlinearity is not essential. We also neglected cell inertia and employed the overdamped limit for the momentum equation \((D\vec{v}/Dt \approx 0)\).

The analysis simplifies considerably in the case of narrow channel flow (small \( x \) dimension), where a finite-dimensional, nonlinear ODE is sufficient to explain the dynamics. The incompressibility condition (5.3) stipulates that \( v_z = v_0 + \alpha z \) where \( v_0 \) can in general be function of time. While the short \( x \)-dimension prohibits the stream-
ing instability, the narrow channel flow in an open trap exhibits a variety of dynamic regimes, including symmetric flow \((v_0 = 0, v_0 = \text{const} \neq 0)\), asymmetric flow \((v_0(t) \text{ is periodic})\), and oscillatory flow \((v_0(t) \text{ is periodic})\), depending on parameters (see Fig. ?? for the results of the bifurcation analysis of the system using MATCONT continuation software).

Non-stationary asymmetric flow regimes have indeed been observed in the open-trap experiments (Fig. 7.18). It is interesting to note the possibility of bistable regimes (e.g. bistability between oscillations and uniform flow). In contrast to the open trap, a side trap has one globally stable solution fixed by the unique velocity profile \(v = \alpha z\).

The existence of cell streaming in the full two-dimensional model was determined by the linear stability analysis of the transversally uniform flow in a side trap geometry (see Fig. 5.2) with respect to small periodic transversal perturbations. We found a finite-wavenumber streaming instability typically near the wavenumber \(k \approx 2L_z^{-1} (L_z \text{ is the distance from the opening to the back wall of the side trap})\). This instability requires the presence of sharp gradients in the friction field \(g(f)\), resulting e.g. from the stationary size function \(c(z)\) varying sufficiently rapidly between the inner and outer boundary of the trap. Intermediate values for the relaxation rate (i.e. \(\gamma \approx \alpha\)) also are required for the instability, such that adaptation to the chemical microenvironment occurs on a timescale comparable to cell division time. Finally, the streaming instability was found to be sensitive to the value of the coefficient of viscosity \(\mu\). However, the effect of finite \(\mu\) diminishes with the size of the trap. For additional details of the model and analysis see Appendix for Chapter 5.

**Discrete-element simulations**

The two-dimensional continuum analysis of streaming addressed only the linear stability of uniform flow. In addition, the continuum model does not include granular effects (Jaeger et al., 1996), including cell shape (Young, 2006; Tripathy and Schweizer, 2009). We therefore performed discrete-element simulations (DES’s) of growing and dividing rod-like cells in a two-dimensional monolayer (Volfson et al., 2008) (see Ap-
Figure 5.3: Space-time diagrams of the narrow-channel flow. DES simulations were started from a single cell placed in the middle of the trap (narrow width $L_x = 10$, length $2L_z = 80$). Color characterizes the cell “diameter” $f$ averaged within a strip of width 2 along $z$ dimension. **a.** Stationary asymmetric regime is seen for parameters $\gamma = 0.5$, $c(z) = 1 + 20 \left( z/L_z \right)^4$; **b.** Oscillatory behavior is seen for parameters $\gamma = 0.1$, $c(z) = 1 + 100 \left( z/L_z \right)^4$. Both simulations have parameters $\ell_{\text{div}} = 3$, $\alpha = 0.5$; **c.** Space-time diagram of $f$ for the continuum description of the narrow-channel flow with the same parameter values.
pendix for Chapter 5 for the details of the computational algorithm and Movies S5-S7). We introduced the cell diameter $f$ as an internal variable carried with each “cell” and inherited by its offspring. The variable $f$ for each cell obeys a linear equation analogous to Eq. 5.2. Cells grow at a rate proportional to their length and divide on average at the length $\ell_{\text{div}}$. Escape of cells from the trap is treated by removing cells with centers that cross the open boundary of the trap.

Figure 5.3 presents space-time plots of the field $f$ for narrow channel flow. In addition to uniform flow (not shown), the narrow channel case can exhibit both asymmetric flow (Fig. 5.3a) and oscillatory flow (Fig. 5.3b, see also Supplementary Movie 5). These results are in close quantitative agreement with the continuum theory (cf. Fig. 5.3b and c), mostly because the otherwise complicating effect of granular viscosity is minor in narrow channel flows.

Simulations for cells in a side trap geometry provide an extension of the linear stability analysis presented above. In the parameter region corresponding to the linear instability we have found significant streaming (see Fig. 5.4 and Movies S6 and S7). Since in most simulations traps of moderate horizontal dimensions (up to 150 cell diameters) were explored, as expected, the granular effects played a significant role in stabilizing streaming instability for cells with relatively small aspect ratios (e.g. $\ell_{\text{div}} = 3$). In particular, we found that the system could be bistable between uniform flow and streaming pattern (see Fig. 5.4b, c). These results demonstrate that the linear instability of uniform flow gives only a sufficient condition for streaming. Similar to experiment, the streams in Fig. 5.4b are non-stationary, allowing drifting, merging, and spontaneous creation of streams. These streams remain dynamic for the entire duration of our simulations.

We probed the effects of cell orientation on cell streaming by analyzing different cell aspect ratios. Longer cells located near streams tend to align their axis along the flow as expected (Volfson et al., 2008). This enhanced ordering locally reduces effective shear viscosity, since aligned long cells easily slide past each other (cell-cell friction is assumed small), and this further increases the intensity of streaming (see Appendix for
Figure 5.4: Streaming patterns in a wide side trap: **a.** A section of a cell population from a typical DES simulation (time $t = 30$ of the simulation shown in panel c). Cells are colored according to their values of $f$. Panels **b** and **c** show $(x, t)$-diagrams of the $z$ component of cell velocity averaged over $z$ axis. Both simulations had the same parameters and were initiated at $t = -15$ with a single cell, but for the simulation but in **b** $f$ was allowed to evolve freely from the very beginning while in **c** $f$ was fixed at $c(z)/2$ until time $t = 5$ and then relaxed. Parameters are $L_x = 150$, $L_z = 20$, $\ell_{\text{div}} = 3$, $\alpha = 0.5$, $\gamma = 0.5$, $c(z) = 1 + 200 (z/L_z)^4$. 
Our theoretical and numerical results indicate that the streaming instability arises due to the strong dependence of cell mobility on its size due to friction with top and bottom of the trap. This would imply that the streaming instability should be sensitive to the depth of the trap. To test this prediction, we performed additional experiments in slightly deeper traps (1.65\(\mu\)m) and indeed, observed the loss of cell streaming (see Appendix for Chapter 5).

**Conclusions**

In summary, we have shown that bacterial monolayers growing in confined spaces are prone to a streaming instability: the outgoing flow of cells may become highly inhomogeneous. While we readily observed the streaming instability in laboratory strains of bacteria grown in microfluidic environments, we believe that the phenomenon is fairly generic and is likely to occur in dense colonies in natural habitats. The mechanism of the instability is related to the coupling between the cell growth and mobility: larger cells experience greater friction force when moving within a confined space. Cell size, in turn, depends on local chemical concentrations of nutrients and metabolic wastes in the environment. In our experiments, cells grow larger near the edge of the trap where the nutrient concentration is higher and waste concentration is lower. The longer the cell remains near the edge of the trap, the larger it becomes and the more difficult it becomes for it to leave the trap. Smaller cells, which are growing in the bulk of the colony, are forced to bypass the larger static cells and form narrow streams. These streams are reminiscent of Saffman-Taylor viscous fingers at an interface between two fluids with different viscosities (Casademunt and Magdaleno, 2000; Bensimon et al., 1986). We expect that similar streaming instabilities will occur in natural environments where cells are attached to surfaces and form dense biofilms. Future investigations of streaming in biofilms may benefit from the inclusion of effects not explicitly included in the present investigation, such as cell-cell and cell-trap adhesion due to an extracellular
polymeric matrix. More generally, the interplay between physical properties of cells and their mobility may play an important role in other examples of morphogenesis, such as, for example, invasive tumor growth (Anderson and Quaranta, 2008).

**Acknowledgements**

This work was supported by the NIH and UC-MEXUS. We are grateful to Dmitri Volfson for writing the original numerical code adapted for our discrete-element simulations, and to Denis Boyer for useful discussions.

Chapter 6

Summary and Conclusion

Synthetic and systems biology propose the rational construction and alteration of the molecular networks that govern life processes, with the purpose of understanding how their architecture relates to the emerging dynamics and for the creation of novel functions. In this thesis I have described our work on the application of synthetic biology, quantitative experimentation and computational simulations to study the processes of entrainment and synchronization of biological clocks, and the dynamics of bacterial growth under mechanical constraints. We have shown how engineered genetic networks can be built and systematically interrogated to explore the rich dynamics that arise when natural clocks exchange information with their environment and with each other. Microfluidics has been instrumental to our efforts, by providing the only means to carry out our experiments, obtain quantitative and abundant data and discover new dynamics. We believe this work may motivate others to further the engineering of biology and to create new technologies to explore biological dynamics in other contexts.
Chapter 7

Appendices

Appendix for Chapter 3

Materials and Methods.

Oscillator strain.

The dual-feedback oscillator is described in full detail in (Stricker et al., 2008a). Briefly, a cycle of the oscillator has two stages, dominated by either protein production or enzymatic degradation. The first stage is a positive feedback driven burst of mRNA from the clock genes lacI and araC, which goes on until enough LacI tetramer repressor accumulates and transcription is turned off, setting the conditions for the second phase where the activator and repressor proteins are enzymatically degraded. Protein degradation brings the system back to a state where a new transcription burst can occur. The time necessary to complete a cycle depends on the magnitude of the activation burst, which is roughly proportional to the induction level of the promoter. Therefore, the natural period of oscillations $T_n$ can be externally tuned through the concentrations of inducers Arabinose and IPTG.

For this work we used the double feedback oscillator strain JS011 (Stricker et al., 2008a). In this strain the genetic oscillator is contained in two plasmids. The activator
and reporter modules are on a derivative of pZE24, a medium-copy ColE1 plasmid, and the repressor module is on a derivative of pZA14, a lower-copy p15A plasmid. These plasmids are transformed into $\Delta araC \Delta lacI$ E. coli to obtain JS011 cells.

**Microfluidics and microscopy**

In the context of synthetic and cell biology, microfluidic devices have been used to obtain abundant dynamic data by lengthening the *in chip* life of microbes (Bennett and Hasty, 2009; Groisman et al., 2005; Balagadd´e et al., 2005; Bennett et al., 2008; Danino et al., 2010, PMID: 20090747). In these designs cells are constrained to microscopic cavities of variable geometry where they are continuously supplied with nutrients. In the case of bacterium *E. coli*, our group and others realized that the mechanical interactions among bacteria and between bacteria and the walls of the micro-cavities leads to cell spatial ordering (Cho et al., 2007; Volfson et al., 2008) (Supplementary Movie 1). Moreover, we previously developed a microfluidic switch that allows the generation of chemical signals of any shape (Bennett et al., 2008).

We built on this work by developing a microfluidic device where bacteria can grow for long periods of time in a dynamic and precisely controlled environment (Fig. 7.1). The device consists of two parts: a trapping region and a dynamic switch. In the trapping region there are 48 rectangular cell chambers distributed in 4 columns. A cell chamber is a rectangular cavity with dimensions 40x50x0.95 $\mu$m$^3$, with the long sides open to the flow of medium. Since *E. coli* cells have a ~1$\mu$m diameter, a trap allows focused observation of the ~400 confined cells. The striped arrangement of cells inside small traps facilitates the exchange of nutrients, inducers and waste between the colony and the surrounding flow. The design also allows continuous discharge of cells into the flow, which carries them outside the device (Supplementary Movie 1). In the dynamic switch, media flowing in from ports I and B (for Inducer and Background) are mixed to generate the modulated signal of inducers. During experiments, the concentrations of small molecules in medium I were $[IPTG] = 2$ mM and $[ara] = 0.3 + A \%$, 

...
and in the background medium B $[IPTG] = 2 \text{ mM}$ and $[ara] = 0.3 - A \%$. Therefore, by changing the hydrostatic pressure at ports I and B sinusoidally we changed the proportion of media I and B in the total flow. Media mixed by diffusion in the long and narrow channel leading from the switch to the trapping region, obtaining the concentration profiles of the signal $[ara](t) = 0.3 + A \sin (2\pi t/T_f)$ % and $[IPTG] = 2 \text{ mM}$.

In preparation for experiments JS011 cells were cultured overnight in LB medium with antibiotics (kanamycin at 100 µg/mL, ampicillin at 100µg/mL). The next day cells are passed into 200 mL of fresh medium in a 1000-fold dilution with antibiotics and average concentrations of inducers ($[IPTG] = 2 \text{ mM}, [ara] = 0.3 \%$). Cells were grown until they reached an OD$_{600}$ of 0.2-0.25. This culture was centrifuged and cells were resuspended in 1.5 mL of fresh medium supplemented with 0.075 % Tween 20 (Sigma-Aldrich), antibiotics and average concentrations of inducers. Cells were introduced to the device from the cell port (C). The hydrostatic pressure at ports W1 and W2 was set so that most cells flowed through the trapping region into W2. No cells were allowed to flow into the dynamic switch area to avoid contamination of the media. Cells were loaded into the traps by gentle flicking of the fluidic line that delivered cell suspension from the reservoir to the chip. To supply loaded cells with media, the hydrostatic pressure at ports C, W1 and W2 was adjusted so that port C became the recipient of the trapping region flow-through and of water flowing from port W1. Water diluted media coming into port C, delaying the clogging of the port by cells. Finally, in order to guarantee oscillators were out of phase before delivering the modulated arabinose signal, cells were allowed to grow inside the traps for 2-3 hours in a constant environment with average inducer concentrations.

In each experiment, the microfluidic device was mounted to the stage of the microscope and primed using a solution of 0.1 % Tween 20 surfactant in water to reduce surface tension and improve wetting. Priming of the device was also necessary to remove all air from the microfluidic channels. This prevented bubble formation when fluid reservoirs were connected to the device inlets and cells were loaded. Culture temperature was maintained at 37º C using a plexiglas incubation chamber. Images were
collected at 100x magnification at intervals of 30 s for phase contrast and 3 min for GFP and MCherry fluorescence. Exposure times were chosen to prevent photobleaching and phototoxicity of cells (100 ms phase contrast, 500 ms GFP, 80 ms MCherry).

**Data analysis**

Ordered bacterial monolayers facilitated the automated tracking of cells and lineage reconstruction. Starting with the original sequence of brightfield images, we cropped each image such that only the cells inside the chamber were visible. We corrected for the positional drift that tends to occur in longer experiments by tracking the location of the frame in the original images. Any out-of-focus images were replaced with a copy of the preceding brightfield image (happens rarely). Potential cell objects were extracted from the cropped images by automatic segmentation by a modified Otsu filter, user preset thresholding, and morphological operations completed in MATLAB (2009b, The MathWorks, Natick, MA). The Otsu threshold filter was used to obtain the background image containing all of the cell objects. Next, morphological opening, erosion, and dilation were applied to the background image to obtain cell object seeds. Using the background and object seed images, CellProfiler(TM) subfunction was utilized as a propagation algorithm to extract individual cell objects. Regionprops command was then employed to collect area, centroid location, and pixel information about each cell object. The goal of cell tracking was to track a cell object from one time lapse image to the next.

Using MATLAB, this was accomplished through a three step process. First, under the assumption that cells did not shift considerably between consecutive time lapse images, two successive images were overlaid and cell objects were matched up based on pixel overlap and distance between centroids. Matches were satisfied based on a one-to-one correspondence. In case of cell division, we were able to match only one of the two daughter cell objects to the mother cell object. In the second step of the cell tracking procedure, division events were identified based on significant reduction in the size of an
object from one image to the next. The second daughter cell object, which did not have a
matching cell object after the first tracking step, was determined based on pixel overlap
and distance from the mother cell object. In the final tracking step, all of the matched
objects from the two successive images were removed and any still unmatched objects
were reassessed based on pixel overlap and centroid distance. Single cell fluorescence
was calculated by averaging the corresponding object pixel values in fluorescence im-
ages. We applied Savitzky-Golay smoothing filter to the fluorescence trajectories before
extracting the periods. We defined the period length as the time distance between two
successive peaks. Using image dilation we identified individual peaks and the respec-
tive troughs for each peak. Amplitudes of each peak were taken as the fluorescence
difference between the troughs and the peak. The peaks whose amplitudes fell below a
predetermined cutoff value, were removed.

Each peak in single cell trajectories marks the increase of the phase by $2\pi$ (or
one cycle). Using linear interpolation between peak times, we obtained phase trajec-
tories for every cell. Using a similar approach we calculated the phase trajectory of the
input signal. By taking the difference between the signal and cell phase trajectories,
we obtained the phase difference between the input signal and each cell trajectory as a
function of time. Single cell trajectories that did not start simultaneously with the signal
where shifted to the origin of the time coordinate.

The single cell data obtained from automated tracking and lineage reconstruction
is available online at http://biodynamics.ucsd.edu/downloads. Each folder contains three
types of MATLAB mat files. The file trajectoriesAraMinxy.mat contains fluorescent
trajectory data (fluorescence, area, position) with rows representing cells and columns
representing time points (3min spacing). The name of the file specifies strength of the
driving signal ($A \% (w/v)$), period of the driving signal ($T_f$, 0Min refers to no free run-
ning data), and the number of the colony (up to 4). The file MaxIntensityArray0.15.mat
contains a matrix for the cutoff for the maximum fluorescence value with rows repre-
senting signals with different entrainment period (0min not included), and columns
representing trap number. The file SignalArray0.15.mat contains a matrix with columns
representing time series of arabinose signals (0min not included) and rows representing time points (3min spacing).

Quantitative analysis

Entrainment regions

Any self-sustained oscillator can be characterized by three main parameters: the amplitude \( A \), natural frequency \( \omega_n = 2\pi/T_n \), and the phase \( \phi \). In an autonomous regime, the phase grows linearly with time as \( \phi_n = \omega_n t \). However, when an oscillator is driven by a periodic signal with frequency \( \omega_f \) (or period \( T_f = 2\pi/\omega_f \)), and phase \( \phi_f = \omega_f t \), its phase \( \phi \) is perturbed according to the phase equation \( d\Delta\phi/dt = \omega_n - \omega_f + \epsilon Q(\Delta\phi) \) (Pikovsky et al., 2001). In this equation, \( \Delta\phi = \phi - \phi_f \) is the difference between the phase of the oscillator and the phase of the driving force, and the term \( \epsilon Q(\Delta\phi) \) represents the effect of external perturbation with amplitude \( \epsilon \). Entrainment occurs if \( \epsilon Q_{\text{min}} < \omega_f - \omega_n < \epsilon Q_{\text{max}} \), where \( Q_{\text{min}} \) and \( Q_{\text{max}} \) are the extremes of the \( 2\pi \)-periodic function \( Q(\Delta\phi) \) (Pikovsky et al., 2001). This condition defines a wedge in the plane \((T_f, \epsilon)\) near \( T_f/T_n = 1 \) which is known as the main Arnold tongue. When the oscillator is nonlinear, or the driving signal is non-sinusoidal, the entrainment may also occur near other rational values of \( T/T_f \), however the corresponding higher-order Arnold tongues are much narrower. If the natural frequency distribution occupies the interval \([\omega_n - \Delta\omega, \omega_n + \Delta\omega]\), the entrainment interval will broaden to \( \epsilon Q_{\text{min}} - \Delta\omega < \omega_f - \omega_n < \epsilon Q_{\text{max}} + \Delta\omega \).

Definition of the entrainment index \( \rho \)

The entrainment index is defined as \( \rho = 1 - S/S_{\text{max}} \), where \( S = -\sum_{k=1}^{N} P_k \ln P_k \) is the entropy of the discrete period distribution, \( P_k \) is the normalized occupancy of the \( k^{th} \) bin, \( N \) is the total number of bins, and \( S_{\text{max}} = \ln N \) (Tass et al., 1998).
Computational modeling

Deterministic and stochastic computational models constructed in (Stricker et al., 2008a) were modified to include the periodic modulation of arabinose. The underlying set of biochemical reactions can be found in the supplementary information of (Stricker et al., 2008a). For all computational simulations the nominal values of kinetic constants where the same and as specified here: \(b_a = b_r = 0.36 \text{ min}^{-1}, \alpha = 35, k_{-a} = k_{-r} = 1.8 \text{ min}^{-1}, t_a = 85 \text{ min}^{-1}, t_r = 90 \text{ min}^{-1}, d_a = d_r = 0.54 \text{ min}^{-1}, k_{fa} = k_{fr} = 0.9 \text{ min}^{-1}, k_{da} = k_{dr} = k_{t} = 0.018 \text{ min}^{-1} \text{molecules}^{-1}, k_{-da} = k_{-dr} = k_{-t} = 0.00018 \text{ min}^{-1}, k_{l} = 0.36 \text{ min}^{-1}, k_{ul} = 0.18 \text{ min}^{-1}, \gamma = 1080 \text{ molecules/min}, c_v = 0.1 \text{ molecules}, \lambda = 2.647, \varepsilon = 0.2, N_a = 50, N_r = 25, C_{a}^{\max} = 0.2 \text{ molecules}^{-1}, C_{a}^{\min} = 0.01 \text{ molecules}^{-1}, k_{a1} = 3.5\%, k_{r2} = 1.8 \text{ mM}, c_1 = 2, b_2 = 2, C_{a}^{\max} = 1 \text{ molecules}^{-1}, C_{a}^{\min} = 0 \text{ molecules}^{-1}, k_{r1} = 0.035 \text{ mM}, b_1 = 2, \) [IPTG] is the IPTG concentration in mM, [ara] is the concentration of arabinose in \% w/v.

We calculated the boundaries of the Arnold tongues numerically using the deterministic model of the oscillator. At every value of the driving amplitude we computed the time series of the concentration of AraC \(a(t)\) and extracted the values \(a_n = a(nT_f)\) separated by the driving period. They form iterations of the return map \(a_{n+1} = F(a_n)\) associated with the continuous-time deterministic model. Within the main Arnold tongue, these iterates converge to a fixed point defined by condition \(a_s = F(a_s)\). The 2:1 resonance tongue corresponds to a fixed point of the second iterate of the return map, \(a_0 = F(F(a_0))\). The boundaries of the main Arnold tongue for a given value of the driving amplitude are manifested as bifurcation points at which the fixed point of the corresponding return map becomes unstable. Numerically, we calculated the coefficient of variation of the sequence of 100 iterations \(a_n\) (for the main tongue) and \(a_{2n}\) (for the 2:1 resonance) after the transients died out, and identified the bifurcation points by the values of the driving period at which the CV crossed the nominal threshold value \(10^{-3}\). In principle, there is a countable set of other higher-order Arnold tongues corresponding to other \(n : m\) resonances, however they are very narrow and difficult to identify,
especially in experiments. Outside the Arnold tongues the phase of the oscillator is not 
locked to the phase of the driving signal. The continuous phase drift is the indication of 
the quasiperiodic behavior (Pikovsky et al., 2001). Close to the boundaries of Arnold 
tongues, the phase drift is non-uniform in time: the intervals of almost constant phase 
difference are interrupted by periodic phase slips.

We used the stochastic model to simulate the forcing of the double-feedback 
oscillator when its components are subject to intrinsic noise only. At every value of the 
driving amplitude and period we computed time series of the number of AraC and LacI 
molecules. We smoothed them using a Savitsky-Golay filter and used a threshold value 
to distinguish true oscillation peaks from noise. From time series we obtained the period 
$T$ and the relative phase $\Delta \phi$, which are defined in the same way as for the experimental 
fluorescence time series (see Fig. 3.1C). The resulting probability distributions of the 
period and relative-phase can be compared to our experimental results (Figs. 7.2-7.5).

The deterministic model was also used to simulate the forced oscillations of indi-
vidual oscillators in a colony of 550 cells where extrinsic noise is the only source of 
variability. To model this we assumed the copy-number of the activator and repressor 
plasmids $(N_a, N_r)$, the transcription and translation rates of AraC and LacI $(b_a, t_a, b_r,$ 
$t_r)$ and the rate of degradation $(\gamma)$ of the ssrA tagged proteins by the ClpXP protease are 
distributed normally around their nominal values. The degradation of tagged proteins 
includes the monomer and oligomer forms of AraC and LacI in any folding state and in 
their free or DNA bound states. The coefficient of variation $(\text{CV} = \sigma/\mu)$ of the distri-
butions is set to CV$= 0.15$, which was chosen to best fit our experimental results and is 
close to the experimental probability distribution of the free-running period CV$= 0.18$.

At every value of the driving amplitude and period we computed time series of 
the concentration of AraC and LacI molecules. From time series we obtained the period 
$T$ and the relative phase $\Delta \phi$, which are defined in the same way as for the experimental 
fluorescence time series (see Fig. 3.1C). The resulting probability distributions of the 
period and relative-phase can be compared to our experimental results (Figs. 7.6-7.9).
Figure 7.1: Microfluidic device constructed for this study. The device consists of two parts: the signal generator (dynamic switch) and the trapping region, where 48 cell chambers host the same number of monolayer bacterial colonies.
Figure 7.2: Comparison of the experimental (blue) and stochastic model (red) probability distributions of the normalized relative phase $\Delta \phi / 2\pi$ for a driving amplitude $A = 0.075 \%$. 
Figure 7.3: Comparison of the experimental (blue) and stochastic model (red) probability distributions of the period $T$ for a driving amplitude $A = 0.075\%$. 
Figure 7.4: Comparison of the experimental (blue) and stochastic model (red) probability distributions of the normalized relative phase $\Delta \phi / 2\pi$ for a driving amplitude $A = 0.15 \%$. 
Figure 7.5: Comparison of the experimental (blue) and stochastic model (red) probability distributions of the period $T$ for a driving amplitude $A = 0.15 \%$. 
Figure 7.6: Comparison of the experimental (blue) and deterministic (green) probability distributions of the normalized relative phase $\Delta \phi/2\pi$ for a driving amplitude $A = 0.075\%$. 
Figure 7.7: Comparison of the experimental (blue) and deterministic (green) probability distributions of the period $T$ for a driving amplitude $A = 0.075 \%$. 
Figure 7.8: Comparison of the experimental (blue) and deterministic (green) probability distributions of the normalized relative phase $\Delta \phi / 2\pi$ for a driving amplitude $A = 0.15\%$. 
Figure 7.9: Comparison of the experimental (blue) and deterministic (green) probability distributions of the period $T$ for a driving amplitude $A = 0.15\%$.

Supplementary Movies

Movie S1. Time lapse fluorescence microscopy of JS011 cells in a microfluidic chamber subject to a sinusoidally changing concentration of arabinose with average $[\text{ara}] = 0.3\%$, amplitude $A = 0.15\%$ and period $T_f = 30\text{ min}$, and constant IPTG concentration $2\text{ mM}$. The GFP and MCherry fluorescence are shown in green and red respectively, the phase contrast image is shown in grey. Total time of the movie is $300\text{ min}$ with a sampling rate of one image every $3\text{ min}$.

Movie S2. Time lapse fluorescence microscopy of JS011 cells in a microfluidic trap at constant inducer concentrations $[\text{ara}] = 0.3\%$ and $[\text{IPTG}] = 2\text{ mM}$. The GFP and MCherry fluorescence are shown in green and red respectively, the phase contrast image is shown in grey. Total time of the movie is $360\text{ min}$ with a sampling rate of one image every $3\text{ min}$.

Movie S3. Time lapse fluorescence microscopy of JS011 cells in a microfluidic chamber subject to a sinusoidally changing concentration of arabinose with average
[ara] = 0.3 %, amplitude A = 0.075 % and period T_f = 30 min, and constant IPTG concentration 2 mM. The GFP and MCherry fluorescence are shown in green and red respectively, the phase contrast image is shown in grey. Total time of the movie is 372 min with a sampling rate of one image every 3 min.

Movie S4. Time lapse fluorescence microscopy of JS011 cells in a microfluidic chamber subject to a sinusoidally changing concentration of arabinose with average [ara] = 0.3 %, amplitude A = 0.075 % and period T_f = 15 min, and constant IPTG concentration 2 mM. The GFP and MCherry fluorescence are shown in green and red respectively, the phase contrast image is shown in grey. Total time of the movie is 300 min with a sampling rate of one image every 3 min.

Movie S5. Time lapse fluorescence microscopy of JS011 cells in a microfluidic chamber subject to a sinusoidally changing concentration of arabinose with average [ara] = 0.3 %, amplitude A = 0.15 % and period T_f = 15 min, and constant IPTG concentration 2 mM. The GFP and MCherry fluorescence are shown in green and red respectively, the phase contrast image is shown in grey. Total time of the movie is 300 min with a sampling rate of one image every 3 min.

Movie S6. Example of the cell tracking algorithm applied to E. coli bacteria in a microfluidic chamber described in the supporting online text. Top left panel shows the capability of our algorithm to track three individual cells using bright field images. The top right panel shows fluorescence profiles extracted from the fluorescence images. The bottom plot shows the fluorescent trajectories corresponding to the average fluorescence value for each cell.
Appendix for Chapter 4

Methods

Strains, growth conditions

Three identical transcriptional cassettes for luxI, aiiA, and yemGFP were constructed by replacing a modular pZ plasmid’s promoter (Lutze and Bujard, 1997) (with yemGFP) with the lux operon from the native Vibrio Fischeri operon luxR up to luxI stop codon (Dunlap and Greenberg, 1985). LuxI and aiiA (Thomas et al., 2005) genes were cloned in place of yemGFP and a degradation tag was added to the carboxy-terminal of each. A previously used MG1655 strain of Escherichia coli\(^1\) was transformed with plasmids pTD103luxI/GFP which is (colE1,Kan) and pTD103aiiA which is (p15A,Amp) to create strain TDQS1.

Each experiment started with a 1:1000 dilution of overnight culture grown in 50mL LB (10g/L NaCl) with antibiotics 100\(\mu\)g/ml ampicillin and 50\(\mu\)g/ml kanamycin for approximately 2 hours. Cells reached an OD600 of 0.05-0.1 and were spun down and concentrated in 5mL of fresh media with surfactant concentration of 0.075 Tween20 [Sigma-Aldrich, St.Louis,MO] before loading in a device.

Microfluidics and Microscopy

Images were acquired using an epifluorescent inverted microscope (TE2000-U, Nikon Instruments Inc., Tokyo, Japan), and chip temperatures were maintained at 37°C with a plexiglass incubation chamber encompassing the entire microscope. Phase contrast and fluorescent images were taken at 20x or 60x every 2-5 minutes and focus was maintained automatically using Nikon Elements software.
Plasmid Construction

The pTD103 plasmids were constructed by replacing the promoter in a pZ modular plasmid (pZE21yemGFP-LAA) from XhoI to EcoRI restriction sites (Lutz and Bujard, 1997) with the luxR gene and the luxI promoter amplified via PCR from the native Vibrio Fischeri operon (Dunlap and Greenberg, 1985). The pZ plasmid RBS was kept the same, and luxI or aiiA (from pMAL-t-aiiA, Thomas et al., 2005) genes were cloned in place of yemGFP with the TSAANNENYLALAA degradation tag on the carboxy-terminal (Stricker et al., 2008a). The yemGFP reporter module (luxR gene-luxIp-yemGFP-LAA) was then amplified with AvrII and NheI restriction sites and ligated into the AvrII site following the terminator in pTD103luxI-LAA.

![Diagram of plasmids](image)

**Figure 7.10:** Plasmids for the synchronized oscillator strain TDQS1. Construction of the pTD103 plasmids was done in the modular pZ plasmid backbones in three identical transcriptional modules with the same promoter, RBS, and terminator for each.

Data Analysis

Fluorescence vs. Time curves were obtained by importing fluorescent images into ImageJ and using the 'Intensity vs. Time Monitor' Plug-in to obtain a mean gray value of the entire field of view, and then the background gray value was subtracted (Fig.4.1c 60x magnification, Fig. 4.2a,b 20x magnification). Peak-to-peak values were taken for all period measurements and amplitudes were measured as peak to previous
trough values. The data collected in Fig.4.2c,d was obtained from 20x/60x magnification experiments from the parallelized device (Fig. 7.12b) in different sized traps. Each data point in Fig. 4.2c,d represents between 10-40 peak values averaged. We found that traps downstream of each other had similar period/amplitude measurements and including them in our averages did not significantly alter the mean values but greatly reduced the errors bar values. This showed that traps downstream of one another were only weakly coupled at our flow rates. In Fig., we plot an additional fluorescence trajectory obtained from imaging one of these traps at 60x showing that oscillations exhibit stably over long periods of time.

![Figure 7.11: Stable oscillations in microfluidic device. Fluorescence vs. Time curve obtained for a 100x84 micron trap over the course of ~ 40 hours.](image)

**Space-Time plots**

To create the space-time plot in Fig.4.3b, we averaged a 20 pixel window along the center of the trap (seen in Fig.4.3a) in fluorescent images. To obtain a quantitative measure of cell density we performed the same process on brightfield images. When no cells were present, the mean gray value was darker due to the lighting on the PDMS (polydimethylsiloxane) device, so we manually corrected the blue region in the bottom left of Fig.7.12 where no cells were present. Once cells populated the trap, we found the gray value to give a measure of the density cells (Fig.7.12). The periodicity in the data
(apparent at high time values) is an artifact from the stitching of images in the Nikon Elements software (due to the slight difference in focal planes when the camera moves). We obtained the space time plot for Fig.4.3d by averaging the fluorescence (20 pixel window) along the center of the colony. We stitched together 3 continuous image sets with image frequencies of 4 minutes (1-45), 3 minutes (frames 46-99) and 2.25 minutes (frames greater than 100). In the displayed images, another colony growing from bottom left begins to merge with the main one, and slightly influences the fluorescent front on the left but did not affect the front to the right.

![Space-time plot of density of cells](image)

**Figure 7.12:** Space-time plot of density of cells in Fig.4.3a experiment. Gray value of the brightfield images is plotted as a measure of cell density in the 2000x 100x 0.95µm device. Red indicates higher cell density.

**Microscopy and Microfluidics**

A similar microscope setup was used in (Stricker et al., 2008a), but to maintain temperature at 37°C a plexiglass incubation temperature was used. At 60x, fluorescent images were taken every 3-4.5 minutes which we found to be sufficient to prevent photobleaching (200-500ms exposure, 10% lamp setting). At 20x magnification, fluorescent images could be taken more often (every 2 minutes).
In each device, *E. coli* cells are loaded from the cell port while keeping the media port at sufficiently higher pressure than the waste port below to prevent contamination (Fig. 7.13). Cells were loaded into the cell traps by manually applying pressure pulses to the lines to induce a momentary flow change. The flow was then reversed and allowed for cells to receive fresh media with 0.075% Tween20 which prevented cells from adhering to the main channels and waste ports. For the yeast device experiments (Fig. 4.3c,d), we loaded *E. coli* cells in the main region by not adding surfactant during the loading process.

We initially built the device in Fig. 7.13a to test the synchronized oscillator in three different trap sizes with 1.65±μm high trap regions. We found the 1.65±μm height allowed for better growth of cells presumably because of the additional flow of media into the interstitial spaces (as compared to 0.9-1.0±μm high traps). A parallelized version of the chip with 3 channel heights was constructed to generate different flow rates and trap sizes of (70,84,90,100)×100 ±μm (Fig. 7.12b). We found that the heights of the channels did not greatly affect the period measurements presumably since the relevant parameter is only the flow rate in the plane of the trap, and this did not significantly differ between channel heights. Thus, to alter the flow rates we increased the heights of the media reservoir to create different flow rates. To estimate flow rates, we measured the length of traces of fluorescent beads (1.0 ±μm) upon 100ms exposure to fluorescent light to establish a measure for the average velocity of as a function of height of the media reservoir. We averaged over at least 1000 data points for each to obtain the average velocities in Fig. 4.2c (x-axis), which confirmed that the velocity scales linearly with the pressure difference caused by the height of the media reservoir.

To study spatial temporal behavior of the synchronized oscillator, we designed a microfluidic trap that is 20 times as long (2mm) and 100 ±μm wide as the original traps (Fig. 7.13c). Unlike the traps in Fig. 7.13a,b, the trap is only 0.95 ±μm high and we found this height optimal for seeding cells in the trap. Since the trap lacks any walls it
is open to the flow, it would be difficult to seed cells in a non constraining device. Given the open boundary conditions and the constriction of rod-shaped *E. coli* bacteria to one layer, cells arrange parallel to each other and perpendicular to the edges of the trap. This ordering leads to a very tight packing of a monolayer of cells. Under these conditions, the transport of nutrients, AHL and cell waste happen mainly by diffusion and is less sensitive (but not insensitive) to the flow rate of surrounding media than in the cell traps of devices *a* and *b* below. An example of this is that bursts of fluorescence propagate in both directions of the trap irrespective of the sense of external flow at very high flow rates. In the experiment shown in Supplementary Movie 3, the flow rate was set close to 100 $\mu$m/s to counter the increased adherence of cells after long run times, which we believe might be caused by growing them in media with surfactant Tween 20 after long durations.
Modeling

There has been much work on modeling asynchronous, oscillating cells coming into synchrony in the context of synthetic biology ((McMillen et al., 2002; Garcia-Ojalvo et al., 2004)), though less attention has been focused on gene networks that do not oscillate in individual cells but oscillate collectively ((Ma and Yoshikawa, 2009)). Here we constructed a deterministic model of quorum-sensing gene clock. From the biochemical reactions depicted in Fig. 4.1a, we derived the following set of delay-differential equation model for intracellular concentrations of LuxI ($I$), AiiA ($A$), internal AHL ($H_i$), and external AHL ($H_e$),

\[
\begin{align*}
\frac{\partial A}{\partial t} &= C_A[1 - (d/d_0)^4] P(\alpha, \tau) - \frac{\gamma_A A}{1 + f(A + I)} \\
\frac{\partial I}{\partial t} &= C_I[1 - (d/d_0)^4] P(\alpha, \tau) - \frac{\gamma_I I}{1 + f(A + I)} \\
\frac{\partial H_i}{\partial t} &= \frac{bI}{1 + kI} - \frac{\gamma_HAH_i}{1 + gA} + D(H_e - H_i) \\
\frac{\partial H_e}{\partial t} &= -\frac{d}{1 - d} D(H_e - H_i) - \mu H_e + D_1 \frac{\partial^2 H_e}{\partial x^2} 
\end{align*}
\]

We did not include an equation for LuxR assuming that it is constitutively produced at a constant level. Previous work found that LuxR is under control of the LuxR-AHL complex to produce a higher concentration of LuxR but we did not find this necessary to capture the essential behavior of the synchronized oscillator ((Williams et al., 2008)).

In the first two equations, the Hill function

\[
P(\alpha, \tau) = \frac{\delta + \alpha H^2_\tau}{1 + k_1 H^2_\tau}
\]

describes the delayed production of corresponding proteins, it depends on the past concentration of the internal AHL, $H_\tau(t) = H_i(t - \tau)$. These delayed reactions mimic the complex cascades of processes (transcription, translation, maturation, etc.) leading to formation of functional proteins. The pre-factor $[1 - (d/d_0)^4]$ describes slowing down of protein synthesis at high cell density $d$ due to lower nutrient supply and high waste...
Figure 7.13: Microfluidic Devices constructed for this study. 

a) Device used initially to test the TDSQ1 cells for synchronized oscillations. The dimensions of the traps from left to right are 100x100 µm, 200x50 µm and 150x100 µm, respectively. Traps scaled 300% in this schematic for visualization. 

b) Parallelized version of Device a. Several trap sizes and channel heights could be tested simultaneously. Traps are 100 µm wide and either 70, 84, 90, or 100 µm deep. 

c) Device used for the wave propagation experiments in Fig.4.3a,b. The trap is 2000x100 µm wide.
concentration. Terms proportional to $\gamma_x$ describe enzymatic degradation of proteins and AHL by proteases inside of the cell due to their degradation tags. We model these processes using Michaelis-Menten kinetics. Terms proportional to $D$ describe diffusion of AHL through cell membrane, and the term proportional to $\mu$ models dilution of external AHL by external fluid flow. The cell density ($d$) determines the amount of external AHL and thus affects the AHL decay rate. The factor $d/(1 - d)$ follows from the total mass conservation of AHL inside and outside the cells. Since the flow speed ($\sim 100 \mu$m/sec) is much higher than the typical wave propagation speed ($\sim 10 \mu$m/sec), we neglected the anisotropy imposed by the fluid flow. The last term in equation for $H_e$ describes the diffusion of external AHL.

We used the following experimentally relevant scaled parameters in most of our simulations: $C_A = 1, C_I = 4, \delta = 10^{-3}, \alpha = 2500, \tau = 10, k = 1, k_1 = 0.1, b = 0.06, \gamma_A = 15, \gamma_I = 24, \gamma_H = 0.01, f = 0.3, g = 0.01, d_0 = 0.88, D = 2.5$. We varied the diffusion constant $D_1$ and the external AHL decay rate (flow rate) $\mu$, as well as the cell density $d$. For “bulk” simulations we dropped the diffusion term $\sim D_1$ in equation for $H_e$, and solved the resulting set of ordinary delay-differential equations. For spatio-temporal simulations we replaced the partial delay-differential equations by a one-dimensional array of $N = 200$ systems of ordinary delay-differential equations representing individual “cells” coupled via a second-order discrete diffusion operator $D_1 dx^{-2}[H_{i-1} + H_{i+1} - 2H_i]$ for the external AHL concentration. We used periodic boundary conditions at the ends of the array ($H_1 = H_N$).

In addition to the numerical results presented in the Main Text, we show here the results of additional spatiotemporal simulations. In particular, Fig. 7.14 shows the synchronization of oscillations in cell population with statistically different parameters. As seen from the figure, the coherence of oscillations increases with the diffusion coefficient $D_1$, as expected. In Fig. 7.15 we show the propagation of waves initiated by a localized initial condition ($I_{N/2} = 1$ while all other $I_i = 0$ and $A_i = 0$) for different diffusion constants. Since parameter $\delta$ characterizing the leakiness of the luxI promoter is small ($10^{-3}$), the basal state with $A = I = 0$ is very weakly unstable. Thus, in the
Figure 7.14: Synchronization of oscillations in spatially extended system with diffusion. The parameters \( p \) of each of 200 oscillators were varied around their nominal values \( p_0 \) as \( p = p_0(1 + \eta \xi) \) where \( \xi \) is a random number uniformly distributed between -0.5 and 0.5, and \( \eta \) characterizes the fluctuations magnitude. To illustrate the role of spatial diffusion in mitigating the stochastic fluctuations, we varied \( \eta \) and \( D_1 \): a, \( \eta = 0, D_1 = 0 \), b, \( \eta = 0.1, D_1 = 0 \), c, \( \eta = 0.1, D_1 = 800 \mu m^2/sec \), d, \( \eta = 0.1, D_1 = 4000 \mu m^2/sec \).
absence of AHL diffusion ($D_1 = 0$), while the middle cell begins to oscillate immediately, all other cells are still quiescent (Fig. 7.14a). However, when the diffusion is present ($D_1 \neq 0$), cells influence their neighbours and oscillations propagate in the form of traveling waves in both directions (Fig. 7.15b-d). As seen from this set of space-time diagrams, the wave speed increases with $D_1$. Fig. 5.4d shows that this dependence is well approximated by the formula $V \approx 0.17d_1^{1/2} \mu$m/sec.

Figure 7.15: Wave propagation in the spatially uniform system with different external AHL diffusion rates: a, $D_1 = 0$, b, $D_1 = 200 \mu$m$^2$/sec, c, $D_1 = 800 \mu$m$^2$/sec, d, $D_1 = 4000 \mu$m$^2$/sec,
Supplementary Movies

Supplementary Movie 1. Timelapse fluorescence microscopy of TDQS1 cells at low flow rate in a 100x100μm trap. Fluorescence is shown in cyan hot color table (dark blue low, white high). Total time of movie is 483 min with a sampling rate of one image every 3 min.

Supplementary Movie 2. Timelapse fluorescence microscopy of TDQS1 cells in a 2000 by 100 by 0.95μm open trap showing propagation of AHL at millimeter scale. The brightfield image is shown in gray, and fluorescence is shown in cyan hot color table (dark blue low, white high). Total time of movie is 962 min with a sampling rate of one image every 3 min.

Supplementary Movie 3. Timelapse microscopy of TDQS1 cells at high flow rate in a 100x100μm trap. Fluorescence is shown in cyan hot color table (dark blue low, white high). Total time of movie is 867 min with a sampling rate of one image every 3 min.

Supplementary Movie 4. Zoomed timelapse fluorescence microscopy of TDQS1 cells in a 2000 by 100 by 0.95μm open trap showing close-up of cells and propagation of AHL. The brightfield image is shown in gray, and fluorescence is shown in cyan hot color table (dark blue low, white high). Total time of movie is 962 min with a sampling rate of one image every 3 min.

Supplementary Movie 5. Timelapse fluorescence microscopy of TDQS1 cells in a three dimensional 1000x400x4.0μm trap. The brightfield image is shown in gray, and fluorescence is shown in cyan hot color table (dark blue low, white high). Total time of movie is 636 min with a sampling rate of one image every 2.25-4 min.

Supplementary Movie 6. Simulation of the wave propagation within a uniform population of cells. The oscillations are initiated by a small perturbation in the middle
of the chamber. The space-time diagram corresponding to this simulation is shown in Fig. 5.4e.

Supplementary Movie 7. Simulation of the wave propagation within a growing dense cluster of cells. The oscillations are initiated by a small perturbation in the middle of the initially small cluster. The space-time diagram corresponding to this simulation is shown in Fig. 5.4f.
Appendix for Chapter 5

Experimental Methods

Microbial strain and growth conditions

Before each experiment we cultured non-motile strain of bacteria *E. coli* (Stricker et al., 2008b) in 50mL LB (10g/L NaCl) with antibiotics (100 μg/ml ampicillin(Amp) and 50 μg/ml kanamycin(Kan)) for approximately 2 hours from an overnight culture. Cells reached an OD$_{600}$ of 0.05-0.1 and were spun down and concentrated in 5mL of fresh media with surfactant concentration of 0.075% Tween20 [Sigma-Aldrich, St.Louis,MO] before loading in a device. During the run cells received the same media(w/ 0.075% Tween20) via diffusion and advection, and grew exponentially filling the trapping regions in a monolayer.

Microscopy and image analysis

Images were acquired using an epifluorescent inverted microscope (TE2000-U, Nikon Instruments Inc.). A plexiglass incubation chamber encompassing the entire microscope was used to maintain the constant ambient temperature 37°C. Phase contrast images were taken at 20x or 60x every 1-2 minutes. Stitching of images and auto-focusing were performed by Nikon Elements software. Each image was processed using grayscale morphology techniques in ImageJ and particle-image velocimetry (MatPIV) was used to measure coarse-grained velocity profiles.

Supplementary Experimental Results

In addition to Fig. 5.1d that showed a space-time diagram for the average escape velocity of cells at the bottom edge of the open trap, here we present a similar plot for the the top part of the open trap, Fig. 7.16. It demonstrates that the dynamics on both open ends of the traps are qualitatively similar: cells organize in fast streams and slow
clusters, which shift laterally as the clusters of large stagnant cells change in size and position.

In order to assess the importance of friction of cells with the wall chambers, we compared cell flows in trap with heights 1.0μm and 1.65μm. These traps are schematized in Fig. 7.17 a,b where the semi-closed geometry prevents the flow of media from sweeping cells away, allowing trap height to be larger than 1.0μm. The spatial distribution of vertical velocities in the 1.0μm case is shown in Fig. 7.17c and the corresponding space-time diagram of exit velocities is shown in Fig. 7.17 d. One stream is clearly identified at the middle of the trap with transient shifts in location and magnitude (Supplementary Movie 2). A snapshot of the cell flow in the ~1.65μm trap is shown in Fig. 7.17e and the space-time diagram shown in Fig. 7.17f. Here, cells are pushed out in a nearly uniform flow across the entire trap, and no streaming pattern is observed.

To study the effect of cell size distribution on streaming, we grew a colony of E. coli in a 300x90x0.95 μm³ open trap, which allows for better distribution of nutrients and more homogenous cell sizes throughout the trap (Fig. 7.18, Supplementary Movie 4). Because the trap is smaller, cells do not have time to grow large enough and form clusters near the periphery. Thus, cells leave the colony uniformly on both open boundaries of the trap. Interestingly, the PIV analysis of the experiment shows that the magnitude of the exit velocity on either side of the trap is anti-correlated (see the space-time plots in Figs. 7.18 b and c), which corresponds to the asymmetric regime of cell flow predicted by the continuum theory.

Fig. 7.19 illustrates the cell size dependence on the position within the side trap. In Panel a we show a snapshot of the side trap where the cells in the interior are significantly smaller than ones near the exit. Panel b shows the average cell area as a function of distance from the open side of the trap. As can be observed in this plot, cell area changes by a factor of 2 from the nutrient-rich open edge of the trap to the back wall. The cross section area of cells was measured by dividing the trap in five horizontal sections centered at z = 9, 27, 45, 63, and 81 μm and segmenting images of cells with ImageJ.
Continuum model for expansional cell flow

We consider a continuum model for the growth of a quasi-two dimensional cell colony in a microfluidic cell trap. Two types of cell trap types are used. A “side trap” consists of a solid boundary at \( z = 0 \) and a free boundary (where cells can escape) at \( z = L_z \). An “open trap” consists of two open boundaries at \( z = \pm L_z \).

The “cell fluid” is assumed to be (a) orientation-free (or free of coupling to any cell orientation), (b) incompressible, and (c) uniformly expanding with volumetric growth rate \( \alpha \). Cell fluid is assumed to experience shear viscous stress as well as velocity-dependent friction force through interaction with the top and bottom bounding walls of the trap.

Equations of motion

According to our assumptions, the cell fluid velocity \( \vec{v}(x, z, t) \) (time \( t \)) obeys

\[
\frac{D\vec{v}}{Dt} = -\nabla p - g(f)\vec{v} + \mu \nabla^2 \vec{v} \tag{7.5}
\]

\[
\nabla \cdot \vec{v} = \alpha \tag{7.6}
\]

where \( p(x, z, t) \) is pressure, \( g(f)\vec{v} \) is the drag on the cell fluid, \( f(x, z, t) \) is a field characterizing “cell size” (e.g. some function of diameter), \( \mu \) is a viscosity, and \( D/Dt \) is the convective derivative

\[
\frac{D}{Dt} = \frac{\partial}{\partial t} + \vec{v} \cdot \nabla \tag{7.7}
\]

The friction coefficient \( g(f(x, z, t)) \) in Eqs. (7.5)-(7.6) depends sensitively on the cell size. For cell diameter larger than the vertical height of the chamber, \( g(f) \) should increase rapidly due to contact friction with the bounding walls, so in our model \( g(f) \) is taken to be some nonlinearily-increasing function of \( f \) (the exact from of this function is not essential).

We assume that there exists some function \( c(z) \) that characterizes the chemical microenvironment around a cell, e.g. representing the concentrations of nutrients and wastes. The cell adapt to its microenvironment with a characteristic rate \( \gamma \), such that the
cell “size” $f$ obeys the linear equation

$$\frac{Df}{Dt} = \gamma (c(\vec{r}) - f)$$  \hspace{1cm} (7.8)

To correspond to experiment, $c(z)$ should be chosen to be largest near the open boundaries, where cells are largest.

For the work presented here, we typically suppose that the functions $c(z)$ and $g(f)$ are of the form $c(z) = A + Bz^n$ and $g(f) = f^\ell$, with $A > 0$, $B \geq 0$, $n$ a positive even integer, and $\ell$ a non-negative integer.

Boundary conditions for the cell fluid are as follows. A solid boundary is defined by zero normal velocity and, when $\mu$ is nonzero, zero tangential viscous stress (a “slip” condition). The slip condition expressed for the inner solid boundary of the side trap is

$$0 = \mu \left( \frac{\partial v_x}{\partial z}(z = 0) + \frac{\partial v_z}{\partial x}(z = 0) \right)$$  \hspace{1cm} (7.9)

Open boundaries are defined by constant pressure (chosen to be zero) and, when $\mu$ is nonzero, zero tangential viscous stress. No inward cell flow is permitted at an open boundary, since we assume the region exterior to the trap is devoid of cells.

**Overdamped approximation**

Unless otherwise stated, we will use an overdamped approximation, such that inertial effects are weak. The equations of motion are then

$$\vec{\nabla}p = -g(f)\vec{v} + \mu \nabla^2 \vec{v}$$  \hspace{1cm} (7.10)

$$\vec{\nabla} \cdot \vec{v} = \alpha$$  \hspace{1cm} (7.11)

$$\frac{Df}{Dt} = \gamma (c(\vec{r}) - f)$$  \hspace{1cm} (7.12)

The system of equations (7.10)-(7.12) can be interpreted as a single evolution law for the function $f$:

$$\frac{\partial f}{\partial t} + (\vec{v}\{f\} \cdot \vec{\nabla}) f = \gamma (c(\vec{r}) - f)$$  \hspace{1cm} (7.13)
where the velocity field $\vec{v}\{f\}$ is determined by the field $f$. $v\{f\}$ is defined as the unique solution to Eqs. (7.10)-(7.11). The $f$-dependence of $\vec{v}$ makes Eq. (7.13) a nonlinear evolution law for the single variable $f$.

**Narrow channel flow**

A special type of solution for the cell trap is narrow channel flow, where all dynamical fields are independent of the $x$-coordinate. Such flow tends to arise for a trap with small lateral dimension $L_x$ (where we assume no friction with the side walls $x = 0, L_x$). The equations of motion can simplify dramatically in the case of narrow channel flow, as we now demonstrate.

**General equations**

For the case of narrow channel flow, we only need to consider fields that are a function of $z$. Defining $v$ as the $z$ component of velocity, the equations of motion (including inertia) can be written as

\begin{align}
\frac{\partial v}{\partial z} &= \alpha \tag{7.14} \\
\frac{\partial v}{\partial t} + v \frac{\partial v}{\partial z} &= -\frac{\partial p}{\partial z} - g(f)v + \mu \frac{\partial^2 v}{\partial z^2} \tag{7.15} \\
\frac{\partial f}{\partial t} + v \frac{\partial f}{\partial z} &= \gamma (c(z) - f) \tag{7.16}
\end{align}

The general solution to Eq. (7.14) is

$$v(z, t) = \alpha z + v_0(t) \tag{7.17}$$

for some function of time $v_0(t)$. Substituting Eq. (7.17) into Eq. (7.15) leads to

$$\frac{dv_0}{dt} + (\alpha z + v_0(t))\alpha = -\frac{\partial p}{\partial z} - g(f)(\alpha z + v_0(t)) \tag{7.18}$$

Assuming an open trap (the side trap case is entirely similar), Eq. (7.18) can be integrated over $z$ on $[-L_z, L_z]$ to give

$$2L_z \left( \frac{dv_0}{dt} + \alpha v_0(t) \right) = -(p(L_z) - p(-L_z)) - \alpha G_1(f) - v_0(t)G_0(f) \tag{7.19}$$
where
\[ G_s(f) = \int_{-L_z}^{L_z} dz \, z^s g(f(z)) \] (7.20)

Since \( p(L_z) = p(-L_z) \) in an open trap, Eq. (7.19) implies
\[ \frac{dv_0}{dt} = -\alpha v_0(t) - \frac{1}{2L_z} (\alpha G_1(f) + v_0(t)G_0(f)) \] (7.21)

In the limit of high friction, Eq. (7.21) reduces to the condition
\[ v_0(t) = -\alpha \frac{G_1(f)}{G_0(f)} \] (7.22)

which defines the flow field for a known friction field \( f(z, t) \).

**Polynomial representation of \( f \)**

The equations of motion (with inertia) reduce further in the case that \( f(z, t) \) and \( c(z) \) are polynomials in \( z \). Suppose
\[
\begin{align*}
  f(z, t) &= \sum_{n=0}^{N} f_n(t) z^n \\
  c(z, t) &= \sum_{n=0}^{N} c_n z^n
\end{align*}
\] (7.23)

where many \( c_n \) may be zero (e.g. \( c_n = 0 \) for \( n > 4 \)). Then Eqs. (7.16) and (7.17) lead to
\[
\sum_{n=0}^{N} \frac{df_n}{dt} z^n + (\alpha z + v_0(t)) \sum_{n=0}^{N} f_n n z^{n-1} = \gamma \sum_{n=0}^{N} (c_n - f_n) z^n
\] (7.24)

By identifying corresponding coefficients, we finally arrive at the set of ODEs
\[
\begin{align*}
  \frac{df_n}{dt} &= -n\alpha f_n - (n + 1)v_0(t)f_{n+1} + \gamma(c_n - f_n) , \quad 0 \leq n < N \\
  \frac{df_N}{dt} &= -N\alpha f_N + \gamma(c_N - f_N)
\end{align*}
\] (7.25, 7.26)

Equations (7.21) and (7.25)-(7.26) provide a closed set of ODEs for narrow channel flow.
Notice that high order coefficients of $f$ remain zero if initially zero. That is, if $c_n = 0$ and $f_n(t = 0) = 0$ for $M \leq n \leq N$, then $f_n(t) = 0$ for $M \leq n \leq N$.

For the side trap geometry ($v_0 = 0$), all $f_n$ decouple from one another. Equations (7.25) and (7.26) can then be used to show that symmetric flow is globally stable.

**Fixed points of Equations (7.25) and 7.26**

The fixed points of the Eqs. (7.25), (7.26) satisfy

\[
(n\alpha + \gamma)f_n + (n + 1)v_0 f_{n+1} = \gamma c_n , \quad 0 \leq n < N \tag{7.27}
\]

\[
(N\alpha + \gamma)f_N = \gamma c_N \tag{7.28}
\]

Equations (7.27), (7.28) can be solved iteratively from index $N$ downwards, providing solutions for $f_n$ as a polynomial in $v_0$. For a given $v_0$, there exists a unique fixed point solution for $f$, $f_n(v_0)$. However, the choice of $v_0$ must be self-consistent with the constraint (from Eq. (7.21))

\[
-2L_z\alpha v_0 = \alpha G_1(f) + v_0 G_0(f) , \quad \text{with inertia} \tag{7.29}
\]

or in the overdamped limit,

\[
0 = \alpha G_1(f) + v_0 G_0(f) , \quad \text{overdamped} \tag{7.30}
\]

One can show that for $g(f)$ proportional to an integer power of $f$, e.g. $g(f) = h f^2$, Eq. (7.29) reduces to solving the roots of a polynomial in $v_0$. Self-consistent solutions to Eqs. (7.27), (7.28) are restricted to have $v_0$ in the physical limits $-\alpha L_z \leq v_0 \leq \alpha L_z$, such that no inward cell flow occurs.

From earlier discussion, we assume that $c_n = 0$ for odd $n$, such that that the problem is symmetric about $z = 0$. In this case, the solution $v_0 = 0$ always exists, and the solutions for $v_0 \neq 0$ occur in symmetric pairs.

**Jacobian of polynomial dynamics**

It is shown here that the high order coefficients for $f$ are not important when studying the stability of fixed points. Since we study bifurcations for narrow channel
flow using low dimensional truncations, it is important that higher dimensions are not important when considering eigenvalues. Overdamped dynamics are assumed in the following discussion.

Suppose that the polynomial $c(z)$ is of even order $M > 0$, and suppose the total system has dimension $N > M$ (including the dynamics of the $z^0$ coefficient of $f$). The Jacobian matrix $J_{n,m} = \partial \dot{f}_n/\partial f_m$ can be derived from Eqs. (7.25)-(7.26)

\[
J_{n,m} = -(n\alpha + \gamma) \delta_{n,m} - (n + 1) v_0 \delta_{n+1,m} - (n + 1) f_{n+1} \frac{\partial v_0}{\partial f_m}, \quad n < N
\]

\[
J_{N,m} = -(N\alpha + \gamma) \delta_{n,m}
\]

where $\delta_{n,m}$ is the Kronecker delta. At a fixed point, $f_{n+1} = 0$ for $n \geq M$. About a fixed point, the Jacobian is upper triangular for $n \geq M$

\[
J_{n,m} = -(n\alpha + \gamma) \delta_{n,m} - (n + 1) v_0 \delta_{n+1,m}, \quad n \geq M \quad \text{(at a fixed point)}
\]

such that $J_{n,m} = 0$ when both $n > m$ and $n \geq M$. An illustration of this matrix is in Fig. 7.20. This partial upper triangular property of the Jacobian implies that the eigenvalues of the Jacobian at a fixed point can be separated into two sets: (1) eigenvalues $\lambda_n = -(n\alpha + \gamma)$ for $n \geq M$, and (2) eigenvalues of the $M$-dimensional block matrix $J_{n,m}$ for $0 \leq n < M$ and $0 \leq m < M$.

Finally, notice that if we only consider side trap solutions ($v_0 = 0$), then the eigenvalues are $\lambda_n = -(n\alpha + \gamma)$ for integers $n \geq 0$. This result can more simply be derived from Eqs. (7.25)-(7.26).

**Local bifurcation analysis of narrow channel flow**

Section on the Jacobian provides evidence that the low-dimensional reduced system (composed of the lowest order coefficients of $f$) is sufficient to analyze bifurcations in the limit of overdamped narrow channel dynamics. Indeed, the high order coefficients decouple if they are initially zero. We explored bifurcations for a particular low dimensional representation of narrow channel dynamics (see Fig. 7.21), and we found a variety of behaviors, including asymmetric steady flow, periodic oscillations.
Richness of the dynamics in the narrow channel limit implies complexity for the general system, even before streaming instabilities are considered. For this reason, we will investigate streaming in the side trap geometry, where the narrow channel dynamics are trivial.

**Linearized equations for small perturbations about zeroth-order solution in a side trap (overdamped approximation)**

Streaming is simplest to analyze in the case where narrow channel-like asymmetric instabilities are forbidden by geometric constraints. This can be done by analyzing a side trap, where instead of two open walls at \( z = \pm L_z \), there is an open wall at \( z = L_z \) and a solid wall at \( z = 0 \). In the following, we present a brief derivation of the equations governing eigenfunctions in a side trap geometry. These equations can be investigated with a mathematical analysis package capable of solving boundary value problems. We do this using the program Maple (version 11). Solutions are first extended from \( z = 0 \) to \( z = \epsilon \) by a high order, but approximate, polynomial solution, in order to avoid singular behavior of the solution near the solid wall. Maple solves the boundary value problem with this polynomial-extended boundary condition.

**\( \mu = 0 \) linearized equations**

First consider the case of negligible granular viscosity, i.e. \( \mu = 0 \). We assume that the zeroth-order solutions, \( v_0(z) \) and \( f_0(z) \), are perturbed by the functions

\[
\begin{align*}
\tilde{v}_z(x, z, t) &= e^{\lambda t} e^{ikx} v(z) \\
\tilde{v}_x(x, z, t) &= i \frac{\lambda}{k} e^{\lambda t} e^{ikx} \frac{\partial v}{\partial z}(z) \\
\tilde{p}(x, z, t) &= e^{\lambda t} e^{ikx} p(z) \\
\tilde{f}(x, z, t) &= e^{\lambda t} e^{ikx} f(z)
\end{align*}
\]

Note that \( \partial \tilde{v}_x / \partial x + \partial \tilde{v}_z / \partial z = 0 \), such that the full divergence \( \nabla \cdot (\tilde{v}_0 + \tilde{v}) = \alpha \). The linearized equations that govern the growth of perturbations are straightforward to de-
rive, with the result

\[ g(f_0) \frac{\partial v}{\partial z} + k^2 p = 0 \]  \hspace{1cm} (7.38)
\[ \frac{\partial p}{\partial z} + g'(f_0) f v_0 + g(f_0)v = 0 \]  \hspace{1cm} (7.39)
\[ v_0 \frac{\partial f}{\partial z} + (\gamma + \lambda) f + v \frac{\partial f_0}{\partial z} = 0 \]  \hspace{1cm} (7.40)

where \( g'(f) = \frac{dg(f)}{df} \). Equations 7.38-7.40 must satisfy the boundary conditions

\[ v(0) = 0 \]  \hspace{1cm} (7.41)
\[ f(0) = 0 , \ (\lambda \neq -\gamma) \]  \hspace{1cm} (7.42)
\[ p(L) = 0 \]  \hspace{1cm} (7.43)

The boundary condition in Eq. (7.42) follows from Eq. (7.40) if \( \lambda \neq -\gamma \), since it can be expected that \( v_0(\partial f/\partial z) + v(\partial f_0/\partial z) = 0 \) at \( z = 0 \). \( f(0) \) may be nonzero if \( \lambda = -\gamma \) exactly, but because these eigenfunctions are always stable, they are not relevant to cell streaming.

Eigenfunctions for the side trap geometry can be associated with the eigenfunctions for the open trap geometry with symmetric uniform flow as the zeroth order approximation. The boundary conditions are then \( p(\pm L_z) = 0 \) and \( f(0) = 0 \) (when \( \lambda \neq -\gamma \)). Symmetry of the open trap eigenfunctions satisfying Eqs. (7.38)-(7.40) is chosen such that \( v_0 \) and \( f_0 \) are odd and even, respectively, with respect to \( z \). This choice ensures that \( v(0) = 0 \), as is necessary for a side trap.

\( \mu = 0 \) lowest order solutions for \( k \rightarrow 0 \)

Instead of \( v(z) \), consider the scaled function \( w(z) = v(z)/k^2 \). The boundary condition for \( w(z) \) is \( w(0) = 0 \). Then Eqs. (7.38)-(7.40) can be rewritten

\[ g(f_0) \frac{\partial w}{\partial z} + p = 0 \]  \hspace{1cm} (7.44)
\[ \frac{\partial p}{\partial z} + g'(f_0) f v_0 + k^2 g(f_0)w = 0 \]  \hspace{1cm} (7.45)
\[ v_0 \frac{\partial f}{\partial z} + (\gamma + \lambda) f + k^2 w \frac{\partial f_0}{\partial z} = 0 \]  \hspace{1cm} (7.46)
In lowest order in $k^2$ (assuming $w$ is order 1) these equations are

\begin{align}
g(f_0) \frac{\partial w}{\partial z} + p &= 0 \quad (7.47) \\
\frac{\partial p}{\partial z} + g'(f_0) f v_0 &\approx 0 \quad (7.48) \\
v_0 \frac{\partial f}{\partial z} + (\gamma + \lambda) f &\approx 0 \quad (7.49)
\end{align}

Analytic solutions for the $k = 0$ case can be indexed by a nonnegative integer $m$, such that

\begin{align}
\lambda_m &\approx -(\gamma + m\alpha) \quad (7.50) \\
f_m(z) &\approx z^m \quad (7.51) \\
p_m(z) &\approx \alpha \int_z^L dz_1 g'(f_0(z_1)) z_1^{m+1} \quad (7.52) \\
w(z) &\approx -\int_z^2 dz_1 \frac{p(z_1)}{g(f_0(z_1))} \quad (7.53)
\end{align}

These eigenvalues $\lambda_m$ for large $m$ are related to those discussed in Section 7. Notice that $m$ should be even if $f$ is to have even symmetry.

**Linearized equations for non-zero viscosity**

The condition $\mu = 0$ is somewhat unrealistic for a granular flow. While $\mu \neq 0$ effects do not appear for narrow channel flow, we find that stability of uniform flow is significantly increased by a small value for $\mu$.

Linearization of the dynamics for $\mu \neq 0$ can be done as in the $\mu = 0$ case. The equations analogous to Eqs. (7.44)-(7.45) are now

\begin{align}
g(f_0) \frac{\partial v}{\partial z} + k^2 p - \mu \frac{\partial}{\partial z} \left( \frac{\partial^2 v}{\partial z^2} - k^2 v \right) &= 0 \quad (7.54) \\
\frac{\partial p}{\partial z} + g(f_0)v + g'(f_0) f v_0 - \mu \left( \frac{\partial^2 v}{\partial z^2} - k^2 v \right) &= 0 \quad (7.55)
\end{align}

The boundary conditions in Eqs. (7.41)-(7.43) continue to apply. Additionally, we assume a continuous tangential stress condition (a slip condition) at both the inner wall
and the outer free boundary, which can be written in this case as

$$\frac{\partial \tilde{v}_x}{\partial z} + \frac{\partial \tilde{v}_z}{\partial x} = 0$$  \hspace{1cm} (7.56)

For the velocity function $v(z)$, this condition leads to boundary conditions

$$k^2 v(0) + \frac{\partial^2 v}{\partial z^2}(0) = 0$$  \hspace{1cm} (7.57)

$$k^2 v(L) + \frac{\partial^2 v}{\partial z^2}(L) = 0$$  \hspace{1cm} (7.58)

Calculations reported in the main text solve these equations using solutions that are odd in $v$.

**Additional material on discrete element simulations**

**Details of simulation**

Our basic algorithm for soft-particle simulations of growing and dividing spherocylinders has been described previously (Volfson et al., 2008). It calculates normal and tangential forces between cells based on the overlap of virtual soft spheres centered at the nearest points on the axes of interacting spherocylinders. The motion of the cylinders is obtained by integrating the Newton’s equations using 4th order predictor-corrector scheme, and each cell’s length $\ell$ and $f$-factor are governed by the first-order ODEs associated with each cell. After the cell length exceeds a certain prescribed value $\ell_{\text{div}}$, the cell is replaced by two collinear cells with half its length at the same location.

Cells experience negligible sliding friction from motion against the solid side walls of the trap (defining the $x$ and $z$ boundaries) or against other cells. The time step is $\sim 0.25 \times 10^{-5}$ (AU). The average length of division $\ell_{\text{div}}$ is typically short, i.e. $\ell_{\text{div}} = 3$, but $\ell_{\text{div}} = 5$ is used for the simulations. The actual division length is chosen randomly from a Gaussian distribution with mean $\ell_{\text{div}}$ and coefficient of variation 0.2. Drag force on the cells is proportional to velocity of the cell times the factor $g(f) = 2(\frac{f}{d})^2 M$, with $M = 1 + 1.5(\ell - d)$ the dimensionless mass of the cell, and $\ell$ the current length of the cell.
Most of the other parameters in the simulation are the same as in a previous work, Ref (Volfson et al., 2008). Note that this includes the cell diameter $d$ being set to $d = \sqrt{3} - 1 \approx 0.73$ (AU).

**Comments on the role of cell shape in streaming**

Though cell streaming can be investigated without including the effects of cell shape, simulations suggest that colonies of longer cells are more prone to destabilize into streams. Additionally, streams of long cells tend to be more highly focused. Fig. 7.22a presents space-time diagrams demonstrating streaming of long cells, with a snapshot of the cell configuration appearing in Fig. 7.23a. Figures 7.22b, c and 7.23b, c show the dynamics of short cells for comparison. The full time-lapse movie of the corresponding simulation runs are shown in Supplementary Movies 6 and 7. We conjecture that long rods enhance streaming by (1) reducing granular viscosity by local ordering of cells into flowing layers, and (2) orienting the stress tensor along the direction of streams. A detailed study of this effect will be left to future study.
Figure 7.16: Space-time plot of the average exit velocity calculated over $\sim20 \, \mu m$ near the top edge of the monolayer segment shown in Fig. 5.1c.
Supplementary Movies

Supplementary Movie 1. Time-lapse microscopy of 400 µm-long segment from the 2 mm-long open trap. Images were taken every minute with a 60x objective using phase contrast and stitched together.

Supplementary Movie 2. Time-lapse microscopy of 90x100x1 µm³ side trap. Images were taken every minute with a 60x objective using phase contrast.

Supplementary Movie 3. Time-lapse microscopy of 90x100x1.65 µm³ side trap. Images were taken every minute with a 60x objective using phase contrast. No streaming is observed in this trap because of the higher depth.

Supplementary Movie 4. Time-lapse microscopy of 125 µm-long segment from an open trap. Images were taken every 2.5 min with a 60x objective.

Supplementary Movie 5. Numerical simulation of the oscillating flow in a narrow open channel (see Fig. 5.3b for simulation details)

Supplementary Movie 6. Numerical simulation of the streaming instability of short cells in a wide side channel. (see Fig. 7.22c for simulation details)

Supplementary Movie 7. Numerical simulation of the streaming instability of long cells in a wide side channel (see Fig. 7.22a for simulation details)
Figure 7.17: **a.** Schematic diagram of the microfluidic device with side traps (light blue rectangles) on either side of the main channel (black). The traps have been magnified 300% for visualization. Traps are seeded with cells from the cell loading port. Cells are supplied with nutrients from the media port, and as they escape from the trap, they are transported by the flow to one of the waste ports. **b.** Sketch of one cell trap. Color indicates the cell “size” c. Snapshot of the z-component of velocity overlaid with a phase contrast image of a cell monolayer confined in a 1μm-high side trap. A single ”red” stream is flanked by two clusters of large slow moving cells. Deeper in the trap, cells are smaller and almost immobile. This snapshot corresponds to the frame at time 149 minutes in the Supplementary Movie 2. **d.** Space-time plot of the exit velocity calculated over ∼20 μm strip at the bottom edge of the monolayer shown in Panel c. In this plot, a stream of cells shows up as a horizontal band along the middle. The blue areas around this band represent the flanking slow cells. **e, f.** Plots analogous to c, d, but for a 1.65μm-high side trap (see Supplementary Movie 3). In this case, the friction of cells along the wall is reduced to a minimum, and streaming is not pronounced.
Figure 7.18: a) Magnitude of the vertical component of velocity overlaid with a phase contrast image of a colony in an open trap that is half as wide (~90 μm) as the chamber shown in Fig. 5.1. This snapshot corresponds to time 122.5 min in Supplementary Movie 4. Unlike in other one micron high traps, here cell size seems to be uniform and less affected by any chemical gradients that may exist within the colony, and cells seem to be pushed out without streaming. Space time plots for the average exit velocity at the bottom (b) and top (c) of the trap respectively. The average of velocity was calculated over 20 μm from each edge. These plots show that the escape velocity on both sides of the trap is anticorrelated.
**Figure 7.19:** a. Snapshot of a colony growing in the \( \sim 1 \mu m \)-high chamber described in Fig. 7.17. b. Plot of the average cross sectional area of cells as a function of the distance from the open edge of the trap.

**Figure 7.20:** Schematic view of the Jacobian matrix defined previously in Equations 7.31 and 7.32.
**Figure 7.21:** A local bifurcation analysis of the narrow channel flow (global bifurcations exist, but are not treated in detail).  

**a.** One parameter local bifurcation diagram (in coordinates $A = c(0)$ and $f_1$, the first-order term of the $f$ polynomial). Oscillations appear between Hopf bifurcations $H_1$ and $H_2$, while fixed points corresponding to asymmetric solutions exist left of $H_2$ and between $H_1$ and the saddle-node point $N$. Unstable fixed points exist left of $C$ and between $C$ and $N$. Stable symmetric fixed points ($f_1 = 0$) are right of $C$. Parameters are $\alpha = 1$, $\gamma = 0.3$, $c(z) = A + (z/L_z)^4$, $g(f) = f^2$, $L_z = 1$.  

**b.** A two-parameter local bifurcation diagram for the system in a in parameters $A$ and $\gamma$. Symbols S, A, and O indicate regions with symmetric fixed points, asymmetric fixed points, or oscillations, respectively. Bistable attractors are listed together, e.g. O/S. BT represents a Bogdanov-Takens bifurcation. The dashed line indicates the value of $\gamma$ used in panel a.  

**c.** A wider view of the bifurcation diagram b. The majority of space not belonging to symmetric flow is associated with a pair of asymmetric fixed points. These bifurcation diagrams are derived from local bifurcation analysis in Matcont. Consistent with the appearance of a Bogdanov-Takens bifurcation (Guckenheimer and Holmes, 1983), a global bifurcation analysis is necessary to fully understand the behavior of this system. Numerical investigation confirms the existence of infinite-period homoclinic bifurcations that lead to large-amplitude limit cycles (data not shown).
Figure 7.22: Space-time diagrams for simulations of cell flows in wide side traps ($L_x = 150, L_z = 20$) with different cell aspect ratios. **a.** Streaming flow of long cells (average length 5 at division), **b.** Uniform flow of short cells (average length 3 at division), **c.** Short cells with streaming flow. Panels **b** and **c** correspond to the simulations in Fig. 5.4 of the main text. Other than differing average cell size and elongation rate (the two are balanced to keep the division rate of long cells the same as short cells), the parameters for the simulation in **a** are the same as in **b** and **c**.
Figure 7.23: Snapshots at time $t = 30$ of the three simulations in Fig. 7.22. Green and red represent low ($f = 0$) and high ($f \geq 10$) values of $f$, respectively, for each cell.
Bibliography


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