Developing Methods to Study Dynamic Biological Processes in Single Cells With Microscopy

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Developing Methods to Study Dynamic Biological Processes in Single Cells with Microscopy

A DISSERTATION PRESENTED
BY
Charles James Baker
TO
The Committee On Higher Degrees In Biophysics
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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Developing Methods to Study Dynamic Biological Processes in Single Cells with Microscopy

Abstract

Many aspects of intracellular dynamics are difficult to infer from bulk experiments: cell-cycle specific responses are hidden by averaging over an asynchronous population, spatial aspects are invisible, and many of the perturbations that cells respond to vary greatly between cells due to spontaneous, stochastic events. Some of these challenges can be addressed by using quantitative time-lapse microscopy. Great strides have been made in terms of imaging modalities with higher resolution, and fluorescent probes with improved properties. However, the methods for handling cells have lagged behind. Many assays are low-throughput, keep cells under poorly controlled conditions, are confounded by artefacts (e.g. due to the point-spread function of light when cells are close to each other), struggle to quantify components present in low numbers, and are often limited in terms of imaging individual cells in changing populations. In this thesis, we developed several methods to deal with these technological challenges, and applied these new tools to various biological processes.

First we present a detailed and reproducible protocol for a platform termed MACS–microfluidics assisted cell screening. MACS provides a single device that can be used with a wide-range of cell types from *E. coli* to human red blood cells. With MACS, cells are subject to mechanical pressing from a PDMS valve that can exert controlled pressure on cells. By employing high pressure, MACS
can squeeze some of the water from *E. coli* cells, reducing the cytoplasmic diffusion rate and allowing individual fluorescent proteins to be visualized and counted as punctate spots. It can also be used to force *E. coli* cells to uptake normally impermeable dyes or retrieve specific cells of interest.

Second we introduce a platform based on a modified version of the mother machine that allows us to track lineages of cells as they enter and exit stationary phase multiple times. We show that with proper design, this platform quantitatively reproduces the properties of the changing batch culture. As a proof of principle, we track cells expressing an *rpos* transcriptional reporter in this device through entry and exit from stationary phase. We show that cells entering stationary phase exhibit a mixed ‘timer-adder’ mode of cell size regulation, producing widely distributed stationary phase cell lengths. Upon exit, the cells instead turn into nearly perfect ‘sizers’, quickly returning to the mean length in exponential growth. The individual cells also varied greatly in when they turned off division as they entered stationary phase, with some cells dividing 2-3 more times than others. We found that, as long as cells do not spend too much time in stationary phase, this pattern was perfectly compensated by the opposite effect during exit from stationary phase, where cells that divided more times during entry divided fewer times during exit.

Finally, we applied these tools to study an archetypal, noisy biological system—plasmid replication control—in single cells. We describe a technique, based on the DNA binding protein MalI, that allows plasmids to be visualized as fluorescent spots that, unlike many conventional DNA binding proteins, does not appear to interfere with native plasmid function. Comparing the full distribution of copy numbers we obtain with this method to results obtained using a highly tested replication arrest assay (unpublished), we found a strong agreement for all statistical properties extracted.
Strikingly, given the constraints on noise control for self-replicators like plasmids, both assays show that the copy number distribution of R1 is strongly sub-Poisson, particularly at the left tail of the distribution. Specifically, essentially all cells nearing division length had a copy number of four or higher despite being present in just seven copies on average in those cells. Furthermore, with the MalI counting assay, plasmid localization was analyzed and found to be distributed throughout the cell, with signs of being weakly excluded from the nuclear region. This contrasts with previous studies that observed strong foci only at the poles of the cells; however, these studies relied on methods which we have observed to create localization artefacts.
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To Jackie, the love of my life.
Before I started graduate school, I was talking with one of my friends who was a graduate student at the time. I remember telling him in jest, "I thought graduate students were supposed to be stressed out all the time. You seem too relaxed to be a graduate student." To which, he replied with something to the effect of "that’s because I am not an experimentalist." Now despite this humorous warning, I found myself more than two years into graduate school switching to experimental work. I can say that, at least in my case, his comment was accurate. However, I now understand how it is possible that his statement can be true and at the same time the population of experimentalists has not gone extinct. There is also an immense sense of satisfaction when an experiment goes well and you get to be the first person to observe something new about the world. However, getting to that point is a constant battle, and I would not have made it without the help and support of a large group of wonderful people.

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I’ve spent more time than many will believe [making microscopic observations], but I’ve done them with joy, and I’ve taken no notice those who have said why take so much trouble and what good is it?

Antonie van Leeuwenhoek

Introduction

Cellular processes are governed by probabilistic chemical interactions, often involving low numbers of molecules [1–3]. These interactions occur randomly, producing fluctuations in concentrations that are often transmitted to many other reactions in the cell. Stochastic noise gives rise to genetically identical cells that vary substantially in gene expression profiles. This variation between cells is fundamental, and many processes in cells cannot be properly understood without considering noise
However, to properly study stochastic variation in gene expression across a population, it is by definition necessary to carefully study large numbers of single cells.

Simultaneously fulfilling the requirements of single-cell resolution and studying large numbers of cells is very challenging. Flow cytometry has helped meet the demand of analyzing millions of individual cells from a culture. However, this technique is hampered by low sensitivity, making low-abundance fluorescent proteins essentially impossible to detect. It also lacks detailed spatial information about the localization of a given fluorescent signal inside the cells or even the morphology of the cells themselves [7, 8]. These problems are exacerbated for microbes, since light scattering is poorly correlated with size at this length scale [9], meaning that even normal, high expression proteins are not always captured accurately. On the other hand, microscopy can provide detailed information about cellular morphology, fluorescent signal localization and signal intensity for a wide range of length scales and signal intensities. However, traditional microscopic media, such as slide-coverslips and agar pads, place cells in locally and globally heterogeneous conditions. This can non-uniformly impact not only cellular physiology but also observed fluorescence, since the fluorescent halo effect can magnify the apparent signal from tightly clustered cells. Furthermore, these methods lack the throughput needed to obtain robust statistics on rare events, such as persister formation or plasmid loss which can occur as infrequently as $10^{-6}$ events per cell division [10, 11], or on conditional properties, such as the fluorescent signal for cells in a given size range and phase in the cell cycle.

Developments in microfluidics, particularly in the past fifteen years, have made it possible to observe single cells with sufficient throughput to obtain robust statistics on many noisy, dynamic
biological phenomena in reproducible conditions while still harnessing the resolving power of microscopy [12–15]. These breakthroughs have made it possible to construct sophisticated microfluidic devices with relative ease [15]. However, as the as the complexity of the device increases, often there is a corresponding increase in the number of failure points, difficulty of construction and the number of controls needed to ensure the device does not introduce artifacts or noise into the process being studied. Furthermore, despite tremendous progress, existing microfluidic devices still lacked desirable features, such as visualizing single molecules, allowing for precisely controlled, dynamic growth conditions, and robust lineage tracking of sufficient throughput to observe biologically important rare events, e.g. plasmid loss and persister formation. Therefore, we have focused on two relatively simple chip designs, the mother machine and MACS, which we have carefully engineered and updated to meet these challenges while still preventing artefacts and ensuring robustness [16–22]. In the mother machine, individual cell lineages are imaged and tracked as they grow and divide in narrow trenches while being fed diffusively by a flow-channel running orthogonal to these trenches. For cells to be carefully retained in the device for many generations while dynamic responses are measured, the trench dimensions must carefully tailored to the exact cell morphology for the given growth conditions. In contrast, MACS uses mechanical pressure to hold cells in place in a microscope field of view, providing population snapshots of bulk cultures. It lacks the lineage information provided by the mother machine, but can be used on a wide array of cell morphologies without modification and the mechanical pressure is a useful tool for a variety of biologically interesting tasks, such as reducing the cytoplasmic diffusion rate.
0.1 Microfluidics-Assisted Cell Screening (MACS)

MACS, Microfluidics Assisted Cell Screening, works by pumping a cell culture through a microfluidic flow channel and gently compressing the PDMS ceiling of the channel to trap cells [20, 21]. These cells can then be imaged using microscopy. By repeating cycles of flowing in fresh samples, then trapping cells, thousands of cells can be imaged per hour. The design also provides a means of accumulating cells in the field of view, allowing extremely dilute cultures to be imaged. MACS provides precise control over the pressure applied to cells, with the option of using input pressures higher than 30 psi. When using MACS in a high-pressure mode, the mechanical pressing of the cells was observed to slow down the diffusion of cytoplasmic molecules such as fluorescent proteins, without loss of fluorescence. This allows low-copy fluorescent signals to be detected as punctate integer-number spots with standard TIRF microscopy. Furthermore, compressing E. coli with the PDMS valve can force the cells to uptake dye that is normally not membrane permeable, such as propidium iodide. Unlike many other microfluidic devices, such as the mother machine, MACS is not tailored to a specific cell-type and can be used on a vast array of suspension cultures. It has been shown to work on Escherichia coli, Bacillus subtilis, Mycobacterium smegmatis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, human red blood cells, and even suspensions of mouse embryonic fibroblasts, without modification to the PDMS chip.

In Chapter 1, we present a reformatted version of an article published this year in Nature Protocols which describes the process of building and using the MACS platform. It begins by describing the limitations of existing methods such as flow cytometry, conventional microscopy and existing
microfluidic devices, particularly for measuring low abundance proteins. We provide an overview of the operating principles of MACS, explaining how MACS traps and presses on cells. We then give an overview of the procedures for implementing MACS, including wafer fabrication, chip soft-lithography, construction of liquid handling components, building of electronic control circuitry, and implementing the software interface. Extensions to the basic protocol are described, including integration of external growth chambers and construction of a specialized MACS device for rare cell enrichment. We finish by providing an overview of the applications of this device and a description of typical experimental results obtained using MACS. The step-by-step procedures, which appear before the Anticipated Results section in the original article, have been moved to Appendix A to aide in readability.

### 0.2 Growth Curve Platform

When trying to observe microbial growth, one is faced with trying to balance the seemingly paradoxical requirements of replicating complex, variable, dynamic nutrient conditions often found in natural environments but doing so in a way that is completely reproducible and tightly controlled. For microbes, conventional bulk cultures can provide a reproducible environment in which nutrients are gradually depleted [23]. In contrast, conventional single-cell imaging procedures, such as agar-pads, provide very poorly controlled, heterogeneous environments for growing cells. Therefore, to study dynamic nutrient conditions with agar pads, cells must be cultured in conventional bulk growth flasks, then aliquoted and imaged immediately to minimize the impact of the uncon-
trolled agar pad conditions [24–26]. However, using this procedure it is difficult to obtain sufficient statistics for the dilute culture present in early exponential phase, to sample quickly enough during the rapid transition from exponential growth to dormancy and then to reduce the fluorescent halo effect when imaging dense stationary phase culture. Even if these challenges are overcome, the results only provide population snapshots, not lineage tracking of dynamic changes in single cells. This makes it impossible to correlate single cell dynamics across time-points during the response to nutrient depletion. This lack of lineage information makes it difficult to fully probe the many rare events and broadly distributed responses associated with nutrient starvation, such as bet-hedging, epigenetic phenomena and the formation of persisters [10, 27, 28]. In contrast, microfluidic devices, such as the mother machine, can maintain cells in highly reproducible conditions such that individual cell lineages can be imaged and tracked for many generations [16]. However, to our knowledge, experiments using the mother machine have focused primarily on conditions of steady, exponential growth.

We introduce a platform that allows microscopic observation and tracking of single cells as they experience the tightly controlled nutrient starvation of conventional bulk growth, enter into stationary phase and then return to exponential growth as nutrients are re-introduced. This controlled entry and exit from stationary phase can be performed multiple times during a single multi-day experiment. The basic operating principle of the platform is quite simple: a growing batch culture is pumped into the mother machine and the cells in the trenches are observed as they respond to the nutrient depletion. At any time during the experiment, the input to the mother machine can be switched to fresh media allowing us to observe cells exit stationary phase and return to exponential
growth. Although conceptually simple, the platform is designed to handle conventional batch culture growth in rich media, with active shaking at $37^\circ C$ and thus produces very dense cultures with optical densities (OD$_{600}$) typically exceeding five in stationary phase, and in extreme cases reaching OD$_{600} > 10$. Unlike dilute cultures, these dense cultures cannot simply be pumped directly into the mother machine without modification. Numerous technical challenges have to be overcome to recapitulate the dynamics exhibited by the cells in the bulk culture in the cells growing in the mother machine. Experiments often require continuous imaging for multiple days all while avoiding device clogs, temperature variations, media contamination, exposure of cells to uncontrolled conditions, long lag times from the growth flask to the mother machine, introduction of bubbles into the microfluidic device and loss of cells from the trenches as they change by up to an order of magnitude in volume during the growth curve.

In Chapter 2, we describe this growth curve platform in detail. As a proof of principle we show changes in cell length and $rpoS$ transcriptional activity for individual cell lineages as they dynamically respond to two rounds of entry and exit from stationary phase. We demonstrate the accuracy with which our platform captures growth in bulk culture by comparing the physiology and $rpoS$ activity of cells in our microfluidic device to snapshots of cells taken from bulk during entry and exit from stationary phase. Phase microscopy is then used to observe the growth dynamics of cells with one minute time resolution as they respond to nutrient starvation along the growth curve, a period of starvation in stationary phase followed by re-introduction of fresh media. During exponential growth, we observe the expected adder-model behavior, with cells adding a fixed amount of volume during each cell division regardless of their size at birth. However, during entry to station-
ary phase, the length added during division becomes more correlated to birth length, exhibiting a mixed adder-timer growth dynamic and resulting in stationary phase lengths that are more widely distributed than observed in exponential growth. The exit from stationary phase resembles a nearly perfect sizer model in which the length of a given cell at division is very weakly correlated with its length in stationary phase. During entry to stationary phase, there is great variability in the number of divisions cells perform before halting and entering full dormancy, with some cells performing 3-4 divisions more than others. This results in widely distributed cell lengths in stationary phase, with the smallest cells performing a full extra division compared with large cells on entry. The opposite behavior is observed upon exit from stationary phase, with large cells having already begun dividing before the small cells finish their first division. Strikingly, by tracking the total number of divisions before and after stationarity we see that this behavior completely cancels, with all cell sizes performing essentially the same number of total divisions. Thus there is no cost to the broad distribution of stationary cell lengths in these conditions. However, when cells are maintained in stationary phase for much longer are provided fresh media, small cells are at a significant disadvantage when resuming growth compared with large cells. In the ten hour period of observation after fresh nutrients are provided none of the smallest 10% of cells had divided but over 85% of the largest 10% of cells had resumed active division. However, this dormancy may be an advantage in other situations; with the obvious example being persistence to antibiotics. We used our platform to characterize persistence in a hipA7 mutant strain and were able to observe the rate of persistence with varying durations of fresh media wakeup before antibiotics are supplied.
0.3 Plasmids

Plasmids are a quintessentially noisy biological system [29, 30]. Unlike many other biological processes, noise in plasmid copy number has clear and direct selective consequences [31]. Plasmids are under selective pressure to both reduce noise in copy numbers, as any increase in variance will increase plasmid loss rates, and to avoid imposing a burden on the host cell by maintaining a low mean copy number [31]. However, plasmids are inherently noisy since they are self-replicators present at low copy numbers that can only respond to fluctuations by increasing the rate of their own replication [30]. To control this noise, all natural plasmids employ negative feedback to control copy number [30]. We know that for negative feedback to effectively suppress noise it must reduce delays, employ short-lived inhibitors produced at high rates and avoid signal cascades or long-lived intermediate molecular species [29, 32]. Furthermore, the mechanisms must be metabolically inexpensive to avoid imparting substantial burden on the host cell. Plasmids are extremely well characterized mechanistically, with many of the molecular players involved in replication control being uncovered before 1990 [33, 34]. The observed mechanisms fulfill the expected constraints for negative feedback, with many plasmid replication feedback systems using short-lived, inexpensive small RNA molecules as direct transcriptional and/or post-transcriptional inhibitors of replication [35]. For example, the primary mechanism of plasmid R1 replication control is mediated by a small RNA, CopA, that is expressed from one of the strongest promoters found in nature, is actively degraded with a half-life of approximately 1 minute and results in turnover of its target mRNA in less than 10 seconds [36, 37]. Despite many of the molecular mechanisms having been characterized many
decades ago, unusually, for plasmids the phenomena that is produced by these mechanisms, namely the copy number distribution, has yet to be accurately measured [38]. The reasons for this are manifold, as will be discussed in Chapter 3. However, perhaps the root difficulty is that the properties of interest in plasmid replication control manifest themselves in changes in the distribution and in many important cases the bulk mean provides no information about the underlying phenomena. It therefore requires determining distributions of copy numbers in single cells.

In Chapter 3, we describe a newly developed method for determining plasmid copy numbers. This method, adapted from a design first used to tag the chromosome terminus [39], consists of fusing a fluorescent protein to a protein involved in the maltose inhibition pathway, MalI, and observing the resultant spots. MalI is an unusual DNA binding protein, in that it binds to its own promoter thus halting its own transcription [40]. Therefore, when MalI is fused to a fluorescent protein it creates very low background fluorescence and only requires between 2-10 binding sites to produce clear spots. By comparison, conventional DNA binding arrays such as LacO often contain hundreds of binding sites causing them to interfere with plasmid localization and replication [38].

We use a MalI-mNeonGreen fusion to quantify the copy number of the R1 plasmid, one of the first plasmids discovered and a common model for replication control. We compare these results to those obtained by a previously unpublished method from the lab. We then characterize the localization of these plasmids in the cell. We finish with a discussion of some outstanding questions raised by this method and description of planned future work.
What is true for E. coli is also true for the elephant.

Jacques Monod
flow of this thesis, the step-by-step protocol is reproduced in Appendix A instead of inline with the text.

1.1 Abstract

Studies that rely on fluorescence imaging of nonadherent cells that are cultured in suspension, such as *Escherichia coli*, are often hampered by trade-offs that must be made between data throughput and imaging resolution. We developed a platform for microfluidics-assisted cell screening (MACS) that overcomes this trade-off by temporarily immobilizing suspension cells within a microfluidics chip. This enables high-throughput and automated single-cell microscopy for a wide range of cell types and sizes. As cells can be rapidly sampled directly from a suspension culture, MACS bypasses the need for sample preparation, and therefore allows measurements without perturbing the native cell physiology. The setup can also be integrated with complex growth chambers, and can be used to enrich or sort the imaged cells. Furthermore, MACS facilitates the visualization of individual cytoplasmic fluorescent proteins (FPs) in *E. coli*, allowing low-abundance proteins to be counted using standard total internal reflection fluorescence (TIRF) microscopy. Finally, MACS can be used to impart mechanical pressure for assessing the structural integrity of individual cells and their response to mechanical perturbations, or to make cells take up chemicals that otherwise would not pass through the membrane. This protocol describes the assembly of electronic control circuitry, the construction of liquid-handling components and the creation of the MACS microfluidics chip. The operation of MACS is described, and automation software is provided to integrate MACS control with image
acquisition. Finally, we provide instructions for extending MACS using an external growth chamber (1 d) and for how to sort rare cells of interest.

1.2 Introduction

Flow cytometry has been very powerful in cell biology, allowing researchers to conveniently analyze millions of individual cells growing in standard batch cultures. However, the throughput comes at the cost of low sensitivity, meaning that many low-abundance FPs cannot be reliably detected. Flow cytometry also provides only limited information \[7, 8\], both about spatial localization of molecules and organelles inside cells, and the sizes and shapes of the cells themselves. This is especially problematic for microbes, in which light scattering poorly correlates with size \[9\], making it difficult to interpret data, even for high-abundance proteins.

By contrast, microscopy is capable of detecting cellular components across a wider range of abundances while providing accurate estimates of cell size, morphology and spatial patterns \[41\]. However, throughput is often much lower, making it difficult to accurately determine statistical properties, detect rare cell states or condition the properties of interest to other measured properties (e.g., the distribution of protein numbers given a certain cell size). The amount of data required for conditional analyses can, in fact, increase exponentially with the number of properties measured. For example, breaking down data into four properties and five classes creates \(5^4 = 625\) separate data sets. Some commercial imaging flow cytometers attempt to bridge this technological gap, but they are nowhere close to the image quality and multiplexing capabilities attainable by microscopy. This is
partly due to the brief residence times of cells within the imaging field of view of the charge-coupled device (CCD), and use of long working distance air objectives with low magnification and numerical aperture. Similar to flow cytometers, imaging flow cytometers are expensive and have high maintenance costs, and are therefore typically only available in staffed facilities. By contrast, microscopes are more generally available within laboratories and provide superior data quality, but typical sample sizes are smaller by many orders of magnitude.

Microscopy also has other limitations. Cells often remain under the microscope for such long time periods—especially when gathering statistics—that their properties can change, making it difficult to compare the results to the batch culture. This problem has been partly addressed by using microfluidic growth chambers [42], which have been tremendously successful in the past few years, increasing throughput substantially and sometimes achieving a uniformity of growth conditions that is comparable to batch culture [16]. However, most existing devices are very sensitive to the exact dimensions of the cells [9, 43], and can easily induce stress response or aging. Even when they work perfectly, the results can be hard to validate without an orthogonal method to measure the same single-cell properties (e.g., to make sure that rare events are not increased in frequency because of the growth chamber). In contrast to flow cytometry, it is difficult to isolate individual cells post imaging, requiring highly specialized setups such as laser-capture microdissection. Finally, although microscopy is generally more sensitive than flow cytometry for detecting low-abundance proteins, the fluorescence signal from many FP fusions is still far below the cellular autofluorescence [26, 41]. For instance, in bacteria, a large fraction of the proteome is present in just a handful of copies per cell [42, 44]. These proteins have been very difficult to analyze [16, 45], and include many key reg-
ulatory proteins that greatly contribute to the phenotypic heterogeneity of cells [18]. We recently developed a microfluidic platform, termed MACS for microfluidics-assisted cell screening, to address the limitations of flow cytometry and traditional microscopy [20]. MACS works by injecting cells through a flow channel, and gently compressing the ceiling of the channel. The advantage of this system is that it allows nonadherent cells to be immobilized for high-resolution imaging while allowing optimization of culturing conditions for longer time periods.

1.2.1 Advantages and Limitations of MACS

The MACS setup improves the statistical power and automation capabilities of microscopy while retaining or even increasing the sensitivity of imaging of nonadherent cells. It works for a wide range of cell sizes and shapes without modifying the device dimensions, while keeping cells in the chosen growth medium with minimal perturbations until the moment of imaging. MACS is compatible with the 96-well plate format, and makes it possible to capture exceedingly rare cells in a population. For E. coli, we have further shown that MACS allows us to exert controlled pressure to slightly flatten and widen cells, presumably by expelling some water. The diffusion of cytoplasmic proteins then slows down by orders of magnitude—enough to ensure that FPs do not move away from a diffraction-limited spot during typical exposure times in fluorescence imaging [20, 26]. Thus, the FPs effectively behave as if they were temporarily fixed. As opposed to chemical fixation, this procedure does not quench the fluorescence signal, and makes it possible to accurately identify single FPs in living cells. We previously quantified this effect by applying our method to single FPs, as well as to FP–FP fusions, by counting photobleaching steps [20], and by comparing the results to other
methods [18], showing that the approach makes it possible to accurately count the number of FPs per cell even when cells contain just one or two protein copies [18]. The flattening of cells in the z dimension, and widening in the x–y plane also helps with counting in three ways: (i) by reducing the projected autofluorescence per area unit, and thereby increasing the signal-to-noise ratio for FP detection, (ii) by further separating the proteins from each other to reduce the risk of spatial overlap and (iii) by making it possible to keep the whole cell in focus. Increasing the pressure further compresses the cells enough to make them take up compounds that otherwise could not cross the membrane, and allows evaluation of cell membrane or cell wall mutants.

The simple design of the MACS chips, their straightforward operating principle and the use of well-characterized valves render MACS robust and reproducible. The valves themselves can be actuated millions of times without signs of fatigue [46], and the major limitation for long-term stability of MACS is instead the eventual accumulation of debris at the intersection of the control channel and the flow channel. Therefore, we carried out optimizations for cell loading into the chip, surface passivation, and media preparation to minimize debris; designed cleaning protocols; and fabricated chips with an array of individual control/flow channel intersections to allow switching to new intersections after debris builds up.

Perhaps the greatest limitation of MACS is that unlike other microfluidic platforms, such as the mother machine [16], MACS cannot track lineages of cells as they grow and divide. However, this comes with the advantage that the dimensions do not need to be optimized for cell retention and growth, and that the device therefore does not need to be specifically tailored to a given cell type. MACS also requires cells to be nonadherent, although problems with sticking to glass could poten-
tially be solved by further treating the surface or sample. MACS could also cause problems with cells that are extremely sensitive to mechanical perturbations, although with most samples we have tested this has not been an issue.

The MACS platform is cheap to build and compatible with a wide range of cell types and microscopes. Other bacterial strains, such as *Bacillus subtilis* and *Mycobacterium smegmatis*, different yeast species such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and mouse embryonic fibroblasts and red blood cells in whole blood samples, can also be imaged on MACS without any modifications to the device. Here, we present a more streamlined and standardized platform, including detailed steps for building and using the setup, as well as the code for data acquisition and analysis. Although the experiments described here are carried out on *E. coli* cells, the setup works for a wide range of nonadherent cell types [20].

### 1.2.2 Overview of the Procedures

MACS capitalizes on the polydimethylsiloxane (PDMS)-based, microfluidic, on-chip valve developed by Quake and co-workers [46], and consists of two layers: a flow layer, through which cells are injected, and a control layer that runs above—perpendicular to the flow layer—and can be pressurized to collapse the flow channel ceiling onto the cells. Here, we describe a protocol for fabricating and assembling the flow and control layers of the PDMS MACS chips using soft lithography (Fig. 1.1). In brief, we use UV light (Fig. 1.1a) and patterned transparency masks (Fig. 1.1b) to etch the desired features into a layer of photoresist deposited onto clean silicon wafers to create negative master molds for each layer. We then cast the actual PDMS on each master and align them. To inject cells
or collapse the control layer, we use liquids pushed out from airtight pressure tubes (PTs). Introducing pressurized air into the PT drives out liquid, either into the flow layer or to pressurize the control layer to push the valve downward. The pressure supplied is regulated by two independent digital pressure regulators ($P_{\text{flow}}$ and $P_{\text{control}}$), which serve as input to air solenoid valves ($AV_{\text{flow}}$ and $AV_{\text{control}}$) that can switch the air flow on or off (Fig. 1.2a). MACS is thus based on imaging cells that are passed through flow channels while air pressure is regulated to partially or completely collapse the flow channel ceiling (Fig. 1.2b). Driving flow with air pressure rather than syringe pumps allows for easy streamlining and fast response times. It is possible to incorporate a growth chamber upstream of MACS, allowing for on-demand microscopy coupled to real-time monitoring of population density, e.g., using batch cultures or turbidostats. This is particularly convenient when studying population dynamics of multiple cell types that change in density over time.
In this protocol, we describe the procedures for building and using the MACS platform, focusing on the following aspects: (i) design and fabrication, (ii) operating principles, (iii) integration of upstream growth chambers and (iv) typical applications. In the paragraphs below, we provide de-
tailed descriptions of the different stages, and we provide recommendations on how to adapt the procedures for specific experimental needs.

1.2.3 Microfluidic device design and fabrication

We used AutoCAD to design high-resolution transparency masks (http://www.outputcity.com) for the patterns in the flow and control layers. We recommend maximum resolution for printing this mask, and printing it with the emulsion side down (i.e., features are printed on the nonglossy side), to ensure minimal distances between the printed side of the mask and the photoresist (PR) on the wafer. A detailed, step-by-step protocol is described in PROCEDURE section (Steps 59–85).

The silicon wafers are cleaned by sequential squirting with generous amounts of acetone, methanol and Milli-Q water (Millipore Corporation) while spinning the wafers on a spin-coater. The wafers are dried for 5 min on a 65 °C hot plate before using the spin-coater to deposit positive PR AZ10xt (AZ Electronic Materials) to a height of 10 μm on top of the wafer. The transparency mask is illuminated with UV light to etch the desired pattern in the PR (Fig. 1.1a), and the wafer is subsequently heated to round off the rectangular channels into dome shapes (AKA reflow) with a final channel height of ∼8.5 μm, which is essential for proper valve closure [46]. We then bake the wafer on a hot plate overnight to stabilize the positive PR. We similarly build a master mold for the simpler control layer of the device by spin-coating the negative PR SU-8 2025 (MicroChem) to a 25-μm thickness on a wafer, and applying a UV pattern to it using the transparency mask for the control channels. These steps produce the silicon masters from which the PDMS chips are molded (Fig. 1.1a).

PDMS is mixed from a two-part Silicone Elastomer Kit (Sylgard 184, Dow Corning), consisting
of a base (part A) and a curing agent (part B) in particular weight ratios (part A/part B). To pro-
duce the MACS chips from the masters, we spin-coat 20:1 PDMS monomer/curing agent on the
flow channel master at 1,250 r.p.m. for 45 s to yield an ∼65-μm-thick PDMS membrane. With this
thickness and PDMS ratio, the minimum pressure required to close the valve is ∼5 p.s.i. If even gen-
tler cell handling is required, a thinner membrane can be made to allow the valve to close at lower
pressures. For the control channels, we pour 5:1 PDMS monomer/curing agent onto the control
layer master. After both layers are partially cured at 65 °C for 33 min, we peel the PDMS off the con-
trol layer master, punch holes through the inlets and align the control layer slabs to the flow layer.
We then cure both layers for another 4 h at 65 °C to achieve thermal bonding, and cut out the now
two-layer chips from the flow layer wafer using a new razor blade. We punch holes in the two-layer
chips, and sonicate them in isopropanol for 30 min to remove debris (Fig. 1.1c) produced during hole
punching. We then rinse the chips with Milli-Q water, and sonicate them in Milli-Q water for 30
min before leaving the chips to dry for 4 h at 65 °C. Finally, we plasma-bond the two-layer PDMS
chips permanently to glass coverslips. Freshly bonded chips are kept at room temperature (21–25 °C)
for at least 1 d to regain the native surface properties after plasma treatment.

Basic variations to the protocol can be made depending on the requirements of a given applica-
tion. For instance, for specific modifications, such as those involved in maximizing throughput, it
may be necessary to alter the device dimensions, in which case other parameters (such as pressure
or PDMS membrane thickness or stiffness) also must be changed to maintain optimal performance
[47]. For the stiffer MACS chips used for mechanical slowdown of the diffusion of cytoplasmic
molecules, we leave the chips at 65 °C for 3 d after plasma bonding to the cover glass. Alternatively,
the stiffness of the PDMS membrane can be increased by using an A/B ratio greater than 20:1. In some applications, e.g., when assessing the mechanical integrity of cell wall mutants or squeezing cells to force them to uptake material, even higher pressure is required. For such devices, we produce both the flow and the control channels with 10:1 or even 5:1 PDMS monomer/curing agent ratio, and bond them together using oxygen plasma, resulting in an even stiffer chip.

1.2.4 Operating principle of MACS and automation

When operating a MACS system, the simplest scheme of flowing cells past the field of view (open state), and then simply stopping the flow and collapsing the PDMS valve (closed state) yields extremely poor trapping efficiency due to the rapid displacement of liquid (Fig 1.2b). Therefore, we introduce a third state that we term ‘half-open’, in which we set \((AV_{\text{flow}}, AV_{\text{control}}) = (\text{on, on})\) and then tune \((P_{\text{flow}}, P_{\text{control}})\) such that cells trickle between the cover glass and the PDMS valve as a monolayer, accumulating in the field of view. After tuning the magnitude of \((P_{\text{flow}}, P_{\text{control}})\) to achieve the half-open state, MACS introduces new cells in the open state by setting \((AV_{\text{flow}}, AV_{\text{control}}) = (\text{on, off})\), accumulating cells in the field of view during the half-open state by setting \((AV_{\text{flow}}, AV_{\text{control}}) = (\text{on, on})\) and trapping cells in the field of view in the closed state by setting \((AV_{\text{flow}}, AV_{\text{control}}) = (\text{off, on})\), at which point the cells are imaged (Fig 1.2b). While MACS is in the closed state, the value of \(P_{\text{control}}\) can be temporarily readjusted before imaging, if needed for the experiment; for example, in single-molecule imaging, \(P_{\text{control}}\) is often increased (Fig 1.2c). This process of serially visiting the open, half-open and closed states, and then imaging, is repeated until the required number of images have been obtained. Each cycle typically takes \(\sim 2-15\) s, corresponding to \(\sim 240-1,800\) frames per h.
Figure 1.2: Operating principle of MACS. (a) The basic MACS setup consists of two digital pressure regulators, $P_{\text{flow}}$ and $P_{\text{control}}$, two air solenoid valves, $AV_{\text{control}}$ and $AV_{\text{flow}}$, and two pressure tubes. $P_{\text{flow}}$ ($P_{\text{control}}$) supplies air to the inlet of $AV_{\text{flow}}$ ($AV_{\text{control}}$), which is actuated to start/stop the flow of air into a pressure tube that is connected to the flow (control) channel of the PDMS chip. (b) MACS works by serially visiting three states of the PDMS valve: open, half-open and closed. Once $P_{\text{control}}$ and $P_{\text{flow}}$ are tuned so that cells flow in a monolayer past the field of view (FOV) when $(AV_{\text{control}}, AV_{\text{flow}}) = \text{(on, on)}$, the three PDMS valve states can be achieved simply by actuating $AV_{\text{control}}$ and $AV_{\text{flow}}$. (c) By using higher-pressure or stiffer chips, MACS can be used for mechanical slowdown of diffusion within cells (orange ovals), allowing for the visualization of single cytoplasmic molecules as small as individual proteins (stars). SM, single-molecule.
Compressing cells in this controlled manner can slow the diffusion of cytoplasmic FPs by almost 100-fold in *E. coli* cells, allowing us to detect them on a standard TIRF microscopy setup (Anticipated Results) with minimal perturbations until the moment of imaging, as we extensively characterized elsewhere [20]. Although this can in principle be achieved by simply using higher pressure (as in Fig. 1.2c), we achieved better performance by using stiffer PDMS chips rather than higher values of $P_{\text{control}}$, as using higher pressure increases the risk of delaminating the microfluidic chip.

Debris accumulation can markedly limit the throughput of MACS. To minimize debris, we fabricated in-line filters on the chips, and filtered all buffers and media using 0.22-μm-pore-size filters (Corning). For experiments that do not require the integration of growth chambers (see separate section below), we grow cells in plastic tubes (round-bottom tubes; BD Falcon) instead of glass vials to prevent contaminating the sample with small glass particles, and sonicate the PDMS chips in isopropanol, as described above. In addition, after setting up all connections and just before using the chips, we run a cleaning routine in which all flow interfaces are extensively rinsed to wash away any PDMS debris stuck to the walls of the chip, as well as particulates that may form when making connections between plastic tubing and blunt-end needles (Box 1). These procedures allow us to keep the same chip on the microscope and use it for multiple days.
Having a cleaning routine is essential, not only to remove debris, but also to prevent carryover between successive samples. Thus, the full MACS setup is a modification of the simple design in Fig. 1.2a, which allows for automated rinsing of all the flow paths using 10% (vol/vol) bleach, 10% (vol/vol) ethanol and Milli-Q water (Fig. 1.3). This full MACS setup can be broken down into three modules: the electronic control base, the cleaning reservoir stand and the pinch valve base. We provide designs to laser-cut acrylic bases to lay out the necessary components for each module. The electronic control base contains all the electronic control circuitry, as well as the digital regulators and air solenoid valves. The air solenoid valves are mounted on manifolds that share a common pressure inlet, but can be independently operated to direct the air flow to a specific airtight container. In comparison with the simple scheme in Fig. 1.2a, air solenoid valves have been added to direct air flow to the bottles holding the cleaning fluids on the cleaning reservoir stand; in addition, a valve that is used for the optional integrated growth chamber and optional cell-sorting function has been added.

The pinch valve base, which normally sits on the microscope stage, contains the two PTs used to run the MACS device (Fig. 1.2a) and various pinch valves that direct the flow of liquids. The only modification needed to run the standard MACS protocol depicted in Fig. 1.2a is to close all the pinch valves except PV to chip (Fig. 1.4). Furthermore, when the experiment is finished, the entire sample can be disposed of by opening AV flow and PV to waste, at which point the whole system can be cleaned by opening PV to PT, PV bleach and AV bleach to flow bleach, or, similarly, ethanol or water. Note that pinch valves provide a distinct advantage over other valve designs in that all the liquids, including the cultures, only ever contact tubing that can be easily replaced if needed.
Overview of full MACS setup. Schematic representation of the three modules comprising the standard MACS setup, including the required liquid and air connections. Electronic control base: pressurized air is connected to the inlet of the pressure splitter and fed into two independent digital pressure regulators, \( P_{\text{flow}} \) and \( P_{\text{control}} \). These pressure regulators supply pressurized air to air solenoid valves—mounted on four-valve manifolds—which are electronically actuated to direct the flow of air to airtight pressure tubes or cleaning-liquid reservoirs. Note: the electronic control base also contains an Arduino Uno, a MOSFET board and an electronics breadboard (not pictured). Pinch valve base: fluids from the cleaning reservoirs and the pressure tube are directed through tubing via pinch valves. For instance, by actuating \( P_{\text{chip}} \) and \( P_{\text{waste}} \) and applying pressure to the pressure tube, fluid from the pressure tube can either be dumped directly to waste or sent to the MACS microfluidic chip. Cleaning reservoir stand: holds the wash fluid reservoirs containing Milli-Q water, 10% (vol/vol) ethanol and 10% (vol/vol) bleach. Between samples, the MACS setup is cleaned with cleaning fluids from the cleaning reservoir stand by opening both \( P_{\text{PT}} \) and the appropriate cleaning fluid pinch valve, i.e., \( P_{\text{bleach}} \), and then applying pressure to the reservoir, i.e., by setting \( P_{\text{flow}} > 0 \) and \( AV_{\text{bleach}} \) to on. When cell sorting is required, an additional air solenoid valve is used (called \( AV_{\text{coll}} \)), as well as two additional PDMS valves controlling the air solenoid valves \( AV_{\text{collect}} \) and \( AV_{\text{capture}} \) (not shown). When integrating upstream growth cultures, an optional pinch valve (\( PV_{\text{external fill}} \)) and air solenoid valve (\( AV_{\text{growth}} \)) are used.
Figure 1.3: (continued)
The cleaning protocol consists of sequentially rinsing the system with 10% (vol/vol) bleach, 10% (vol/vol) ethanol and buffer (or Milli-Q water) in two stages (Box 1). In the first stage of cleaning, we fill the PT with 10% (vol/vol) bleach (open PV\text{bleach}, PV\text{to PT} and AV\text{bleach}) (Fig. 1.3), wait for 5 s, empty the PT and repeat the this process. This gets rid of any sample that may be stuck to the walls of the PT. In the second stage of cleaning, we again fill the PT with 10% (vol/vol) bleach, send a small amount of the 10% (vol/vol) bleach to waste (open PV\text{to waste} and AV\text{flow}) and then flow bleach through the MACS chip for $\sim$1.5 min (open PV\text{to chip}). We then repeat the same two-stage cleaning protocol using 10% (vol/vol) ethanol followed by Milli-Q water or buffer. As a demonstration that this cleaning routine is effective, we used MACS to image \textit{E. coli} expressing CFP, ran the cleaning protocol and then imaged \textit{E. coli} expressing RFP. Not a single CFP-expressing cell was detected among $\sim$10,000 RFP-expressing cells imaged, suggesting that the cleaning routine effectively prevents carryover between successive runs of MACS.

1.2.5 \textbf{Integration of growth chambers}

A schematic of a growth chamber incorporated upstream of MACS is shown in Figure 1.5. We suggest paying attention to the aeration needs of the growing culture. This configuration does not necessarily allow for optimal aeration without other modifications, as the rigorous stirring or shaking required for optimal aeration can interfere with OD600 measurements. For experiments that require active aeration or shaking, the OD600 measurements can be separated from the growth flask. A detailed description of this setup is in preparation. With further modifications, it is possible to implement treatment of the samples with drugs or inducers without affecting the rest of the growing
Figure 1.4 (following page): Flow chart for operation of MACS. The protocol begins by running a full cleaning procedure with 10% (vol/vol) bleach, 10% (vol/vol) ethanol and water to clean the PDMS chip and the flow path. Then, using a pipette, 600 μl of a cultured sample is loaded into the flow pressure tube. This sample is injected into the PDMS chip by flowing first to waste for a very brief interval and then flowing into the chip. Next, all the valves are closed before setting the pressures to 20 p.s.i. The AV<sub>to chip</sub> valve is opened to flow cells past the FOV. This is the open state. Next, AV<sub>control</sub> is opened; this puts pressure on the PDMS valve. If the pressures were chosen correctly for the sample, then cells will flow past the field of view as a monolayer and accumulate under the PDMS valve. If this does not happen, the pressures must be readjusted until a monolayer of cells is seen. At this point, the chip is in the half-open state. Finally, after accumulating cells in the half-open state, AV<sub>flow</sub> is closed, and the cells are trapped in the FOV. This is the closed state. If the experiment requires single-molecule imaging, the value of P<sub>control</sub> can be temporarily increased, applying additional pressure to the trapped cells. Next, cells are imaged under the microscope, and if P<sub>control</sub> was adjusted for imaging, it should be returned to its previous value. This cycle of open, half-open and closed, followed by imaging, can then be repeated for as many cycles as needed, without tuning pressures. Once imaging of the sample has been completed, the culture is expelled to waste, and the setup is cleaned, at which point new samples can be loaded and the procedure can be repeated.
Figure 1.4: (continued)

- Run cleaning procedure
- Load sample and inject cells into flow channel
- Close all valves
- Set $P_{\text{flow}} = 20$ psi and $P_{\text{control}} = 20$ psi
- Open $P_{\text{V to chip}}$ and $A_{\text{Vflow}}$
  - Close $A_{\text{Vcontrol}}$
  - Open $A_{\text{Vcontrol}}$
  - Are cells flowing past in a monolayer?
    - no: Adjust $P_{\text{control}}$ and $P_{\text{flow}}$ (see troubleshooting)
    - yes
      - Close $A_{\text{Vflow}}$
      - Need single molecule resolution?
        - yes: Temporarily increase $P_{\text{control}}$ until finished taking image
        - no
          - Image Cells
          - Finished imaging current sample?
cell culture. The growth chamber can be modified to run in specialized modes, such as turbidostat or chemostat mode, by using peristaltic or solenoid pumps.

We believe that this single, simple device serves many purposes—increasing throughput as compared with conventional microscopy; ensuring that cells can be grown in the desired liquid culture until moments before imaging in 96-well plates, if desired; retrieval of rare cells of interest; pressing on cells to achieve accurate mechanical fixation to count individual molecules; and squeezing cells to force them to uptake chemicals that otherwise could not pass through the membrane. Although MACS does not have the advantage of tracking cell lineages that some other microfluidic devices provide, it also does not need to be precisely tailored to the exact cell sizes and shapes of a given sample, as is required to use those devices. In fact, the same device can be simultaneously used for cells that vary by >100-fold in volume. It can also serve as an orthogonal test to determine whether growth chamber methods truly reflect cell growth in suspension cultures.

1.3 Applications

1.3.1 High-throughput imaging

As a proof of concept for the stability and throughput capabilities of MACS, we acquired unattended snapshots of ~10⁶ *E. coli* cells in a 4-h time window at a single valve intersection [20]. Conventional automated x–y stages can achieve a comparable performance in terms of sampling speed, but cells are then kept on the surface for long time periods before imaging, which can change their properties. MACS, on the other hand, maintains cells in the desired medium, and brings in a fresh
Figure 1.5: Integration of growth chambers with MACS. Schematic showing sampling from a growth chamber, in which we can monitor the OD of a growing culture in real time. Passive aeration of the culture is achieved through the normally open pinch valve ($PV_{growth\ vent}$), which is connected to open air by default. To flow from the growth chamber to the PT, we open $AV_{growth}$ and $PV_{external\ fill}$ (Fig. 1.3) and close $PV_{growth\ vent}$. The detector—placed at 180° with respect to the emitter—measures the transmission of light through the sample, which serves as a metric for OD (OD600) after calibration [48].

We typically achieve a throughput of 500–5,000 cells/min, depending on parameters such as magnification, trapping area and cell density. We typically use 250× magnification (100× combined with 2.5×) for single-molecule counting, and 60–100× for high-throughput data acquisition. For larger cells, lower magnifications can be used to retain high throughput. Rather than carrying out distinct cycles of cell trapping and imaging, MACS can also run constantly in the half-open valve state to flow a stream of cells through the field of view (Fig. 1.6a). This sacrifices image quality somewhat.
but can be used to detect rare phenotypes, for example, those in which the readout is fluorescence levels above some threshold, or the presence of a localized signal.

The throughput of MACS is limited by the time between successive rounds of cell trapping and, on a longer time scale, by the eventual accumulation of debris in the field of view. Debris accumulation is problematic only during actuation of the valve and in the presence of sample flow. Unless debris is forcibly adhered to the PDMS by actuating the PDMS valve, it does not become permanently stuck and is eventually washed away. If the intersection used does become filled with debris, one of the many unused intersections on the same chip can be used for imaging, as intersections not actively in use do not collect debris. The time between rounds of cell trapping is typically dominated by the time it takes for cells to stop moving when the channel is in the closed state ($t_{\text{closed}}$).

The cells become nearly stationary on the glass in as little as 1 s, but can exhibit small movements up to ∼10–15 s after the chip enters the closed state. Thus, for single-molecule counting experiments, we often wait 15 s ($t_{\text{closed}} = 15$ s), but we wait only 1–5 s when exact localization of single molecules is not required. The optimal value for $t_{\text{closed}}$ may also display chip-to-chip variability, and it depends on factors such as cell density and cell type.

If the field of view of the camera is small compared with the total cell-trapping area, a substantial speedup can be achieved by using an automated stage and moving the field of view to cover the entire trapping area. When even greater throughput is needed, it is also possible to modify the dimensions of the chip to fabricate larger valves and thus larger cell-trapping areas. In principle, it could also be possible to run and image several intersections simultaneously to increase throughput further.
For a given cell density in the medium, we can also adjust the coverage within the field of view by simply changing parameters such as $P_{\text{flow}}$ and $P_{\text{control}}$, as well as the duration of the half-open state. Modifying cell density is particularly important for the single-molecule counting assay, as it is hard to exert enough pressure on each individual cell to slow diffusion in areas with a very high local density of cells.

Reducing the cell density can also be important, e.g., when carefully quantifying fluorescent levels between cells, as the point-spread function of light can cause light emitted from one cell to be attributed to its neighbor cells. Cells in areas of high cell density can then artificially appear brighter. On the other extreme of low cellular density (e.g., the balanced growth in bacteria), MACS can function as a concentration device because the half-open state can function locally as a 'sieve' and permits gathering of statistics that would not be possible via traditional sample preparations (such as agar pads), which simply spread cells on cover glass surfaces.

1.3.2 Retrieving cells with rare phenotypes

The setup can also be used to screen large numbers of cells, identify rare cells of interest for higher-quality imaging and then retrieve the cells from the field of view, including the cell of interest, and place them into test tubes. This process enriches the ratio of cells with a rare phenotype with respect to the population. For example, it is possible to run MACS in the highest-throughput mode, in which cells are constantly moving through the device and imaged in video mode as they trickle by (Fig. 1.6a), and use real-time image analysis to identify cells of interest. By instantaneously switching to the closed state, these cells can be trapped for high-quality imaging. To illustrate this capability,
we first took a cell culture of an *E. coli* strain expressing GFP and spiked it with a few cells from an RFP-expressing strain. On-the-fly image processing allowed detection of the cell of interest (i.e., a red cell) as it appeared within the field of view (Fig. 1.6b). Although the detection rates depend on multiple parameters such as flow rate, cell density and the closing properties of the valve, in our trial runs, cells diluted at ratios of 1:10,000 and 1:100,000 were detected within, on average, ~3 s and ~30 s, respectively. This allows us to estimate the occurrence probability of rare phenotypes of interest and then characterize those phenotypes with more detailed imaging.

Minor modifications to the design make it possible to retrieve the trapped cells from the device. This allows us to enrich or even directly isolate rare phenotypes (Fig. 1.6c). In this modified design, we control two inlets and outlets via on-chip valves (valves 1–4). During screening, the MACS device is in the half-open state, and cells are imaged as they flow past the field of view to the waste (valves 3 and 4 are closed, and valves 1 and 2 are open). When a rare cell of interest is detected (Fig. 1.6b), cell flow is immediately stopped, thus switching the MACS device to the closed state. At this point, detailed images can be acquired, if needed. Next, we close all inlet and outlet valves (valves 1–4), and release the control valve (Fig. 1.6d). We then open valves 3 and 4, allowing oil to flow into the chip and sending the trapped volume out for collection. After growing the retrieved cells overnight and imaging them on agar pads, we observed that the RFP-expressing cells were highly enriched (Fig. 1.6e,f). Using an oil phase for cell collection provides precise control of the volume that is retrieved and serves to separate the successive collections. To facilitate collection using the oil phase, we treated the chips with a commercial water repellent [49] (Aquapel) after plasma bonding. We kept these chips at room temperature until use.
1.3.3 High pressure MACS for single-molecule imaging and intracellular delivery

Perhaps, the most striking feature of this device is that it allows us to apply pressure to *E. coli* cells in a controlled way, such that the diffusion of cytoplasmic proteins can be slowed by two orders of magnitude [20]. We believe that this reflects the interesting finding that the *E. coli* cytoplasm is close to a glass transition [50], in which slightly compressing cells causes them to expel some of the water from the cytoplasm and appears to cause the movement of cytoplasmic proteins to transition from almost free diffusion to crowding.

Regardless of the underlying mechanism, we have found that the slowdown of diffusion allows for visualization and even accurate counting of FPs at exceptionally low abundances, using conventional TIRF microscopy setups [18, 20]. As opposed to chemical fixation protocols, this mechanical fixation can be achieved with no loss of fluorescence, and extensive controls with photobleaching steps and tandem dimer FPs [20] suggest that we do detect nearly all mature fluorescent molecules.

Practically, we find that the increase in effective pressure is easiest to achieve by using stiffer PDMS chips. Specifically, we recommend keeping the chips at 65 °C for 3 d after plasma bonding to coverslips to achieve enough stiffening to induce cytoplasmic slowdown. Unless the pressure is greatly increased, which can cause other problems, pressing chips that have not been ‘aged’ in this manner does not cause sufficient slowdown to visualize proteins within the typical 10–100-ms integration times of a CCD camera. For single-molecule imaging, it also becomes critical to properly clean the glass surfaces, or else fluorescent background spots can masquerade as actual FPs. We rec-
Figure 1.6 (following page): Capturing rare phenotypes and their retrieval using MACS. (a) Cells continuously flowing through the field of view in the half-open state. Scale bar, 25 μm. (b) The cell of interest—an RFP-expressing cell, which we diluted by a factor of 100,000 using GFP-expressing cells—is captured in the closed state and is detected (circled). Scale bar, 10 μm. (c) Minor modification of MACS enables cell retrieval. Masks for flow (top) and control (bottom) layers. Scale bars, 1 mm. The flow-focusing feature is shown within the semitransparent gray box. This feature is optional, and allows the cells to trickle through the central region of the flow channel and prevent cell accumulation at the channel sides. It also allows adjustment of the cell density on the FOV: when the side streams (of media) are stronger, the middle stream becomes thinner, thereby diluting the cells at the FOV. (d) Schematic of cell retrieval. The volume that is trapped when valves 1–4 are closed (with the control valve open) is shown by the red dashed line. (e) The captured cell of interest in Figure 1.6b (circled) within the trapped volume, which is outlined by the red dashed line. Overlaid bright-field and RFP fluorescence images are shown. Scale bar, 40 μm. (f) When we collected the trapped volume using the oil phase, grew cells overnight and imaged them on the agar pad, the RFP-expressing cells were enriched. This scheme allows for the immediate retrieval of cells at low cell densities. At high densities, a second round is necessary to achieve 100% purity. Scale bar, 2 μm. Image adapted with permission from ref. 12, Nature Publishing Group.
Figure 1.6: (continued)
ommend a cleaning protocol described by Elf et al. to clean glass coverslips [25]. Alternatively, glass coverslips can be kept under oxygen plasma for 10 min. It is also essential to wear gloves throughout, as touching the involved surfaces with bare hands can cause substantial fluorescent impurities. The growth media can also introduce fluorescent particulates. For *E. coli*, we found that the standard LB medium, which is highly fluorescent, can be replaced with M9 media supplemented with 10% (vol/vol) LB without compromising the growth rate; it greatly reduces false-positive counts due to fluorescent impurities. The rich-defined medium EZRDM (Teknova) works even better for this purpose. By paying close attention to cleaning procedures at different stages, background fluorescence can be lowered to as few as 0.3 spots/cell for a control strain that does not express any FP [18].

1.3.4 **Forcing the uptake of small molecules**

Another feature of MACS is the observation that compressing *E. coli* cells in the closed state forces them to uptake molecules. Specifically, when we used extra-stiff chips, made by plasma-bonding two 10:1 PDMS monomer/curing agent layers, we could press harder on cells, causing them to uptake a DNA-staining dye that is normally cell impermeable (propidium iodide; Thermo Fisher) (Fig. 1.7 and Supplementary Video 1). This procedure also allowed us to distinguish wild-type cells from cell wall mutants, as the latter uptake more dye at lower pressures than the wild type.

1.4 **Anticipated Results**

After setting up MACS, one should be able to run automated image acquisition of nonadherent cells, which are temporarily immobilized. For a short segment of an experiment in which cells were
Figure 1.7: Dye uptake upon pressing. The *E. coli* cells are first mixed with propidium iodide and then squeezed by the control-layer valve of the MACS device. (a,b) Top and bottom panels show two different cells in the bright-field channel (a) and propidium iodide fluorescence channel (b) right after pressing. (c) Propidium iodide fluorescence channel 9 min after pressing. Scale bar, 1 μm

Imaged at a rate of ~100,000 cells/h, see Supplementary Video 2. This technique can be applied to count single molecules in bacteria, and to detect, enrich and sort rare phenotypes, applications for which we provide examples below.

For the single-molecule counting measurement, we used an *E. coli* strain in which a low-abundance protein (SprE) had been fused to GFP (mNeonGreen) at its native chromosomal locus. An exogenous copy of a second fluorescent protein (CFP) on the chromosome served as a segmentation
Figure 1.8: Electrical connections for the MACS setup. Commands are sent from a computer to an Arduino Uno microcontroller, which translates the commands into electrical signals. These electrical signals actuate MOSFETs on a MOSFET board (green PCB in the center of the figure) to control the pinch valves and air solenoid valves. A second MOSFET board is used to control the air solenoid valves required for the optional cell-sorting functionality. A 24-V DC power supply should be connected to the DC barrel jack of each MOSFET board. The pressure of $P_{\text{flow}}$ and $P_{\text{control}}$ is set to an analog voltage level between 0 and 5 V. The voltage corresponding to the desired pressure set by the Arduino using external digital-to-analog converters (DACs) controlled over an i2c bus over an i2c bus. These DACs should be connected, as shown in the breadboard (center left). For the optional growth chamber, $AV_{\text{growth}}$, $PV_{\text{growth vent}}$ (normally open) and $PV_{\text{external fill}}$ are actuated by the same MOSFET, so they should be electrically tied together as shown. Fritzing was used to make the figure. A0, i2c address selection pin; GND, ground; i2c, inter-integrated circuit; SCL, serial clock line; SDA, serial data line; VDD, positive supply voltage; VOUT, analog voltage out.
Figure 1.9: Electrical connections for the MACS setup. Commands are sent from a computer to an Arduino Uno microcontroller, which translates the commands into electrical signals. These electrical signals actuate MOSFETs on a MOSFET board (green PCB in the center of the figure) to control the pinch valves and air solenoid valves. A second MOSFET board is used to control the air solenoid valves required for the optional cell-sorting functionality. A 24-V DC power supply should be connected to the DC barrel jack of each MOSFET board. The pressure of $P_{\text{flow}}$ and $P_{\text{control}}$ is set to an analog voltage level between 0 and 5 V. The voltage corresponding to the desired pressure set by the Arduino using external digital-to-analog converters (DACs) controlled over an i2c bus over an i2c bus. These DACs should be connected, as shown in the breadboard (center left). For the optional growth chamber, $AV_{\text{growth}}$, $PV_{\text{growth vent}}$ (normally open) and $PV_{\text{external fill}}$ are actuated by the same MOSFET, so they should be electrically tied together as shown. Fritzing was used to make the figure. A0, i2c address- selection pin; GND, ground; i2c, inter-integrated circuit; SCL, serial clock line; SDA, serial data line; VDD, positive supply voltage; VOUT, analog voltage out.
marker and allowed the identification of cells and their boundaries (Fig. 1.10). For this experiment, we used chips that had been cured for 3 d at 65 °C after fabrication (Step 57), and thus the chips were stiffer. Cells were injected, accumulated in the half-open state and trapped with an imaging pressure ($P_{\text{control}}$) of 30 p.s.i. Approximately 15 s after trapping, cells were imaged using a 50-mW 514-nm laser with highly inclined and laminated optical (HILO) modality on an electron-multiplying CCD (EMCCD) camera at video rate (33 Hz; i.e., 30 ms of exposure time). Details of the setup are fully described elsewhere [20]. We then used the provided MATLAB code, AnalyzeMacsInteractively.m (Supplementary Data 9), to analyze the data; see the Supplementary Tutorial for a detailed description of using the code. Under these growth conditions, the average number of SprE molecules per cell was $\sim$14, and the population-wide distribution of SprE abundance is close to Poissonian.

Running MACS in the continuous mode, we detected *E. coli* cells that are proficient in plasmid conjugation—a leading cause of horizontal gene transfer and the spread of antibiotic resistance genes. As the expression of transfer (tra) genes is associated with higher metabolic burden and increased sensitivity to male-specific phages, many conjugative plasmids are naturally repressed [51]. For example, under laboratory conditions, it is known that plasmid R1 turns on the conjugation machinery at frequencies $<10^{-3}$ per cell and per generation [52], but even order-of-magnitude estimates have proven challenging because the events are so rare. To identify these rare events, we placed a cfp gene at the very end of the transfer operon, and used MACS to identify cells expressing CFP (data not shown). Our preliminary data suggest that the tra operon is activated at a frequency of $\sim10^{-6}$ per cell and generation, under our conditions.
Figure 1.10: (a) The analysis code overlays the outline of the cell boundary, and detected spots are shown as non-red-colored dots (purple in this case). Spots not assigned to any cell are shown as red dots; in this case, the spots are single molecules of SprE-mNeonGreen. The left image shows a snapshot of the cell constitutively expressing CFP, a protein that is used to define the cell boundary and thus segment the cell. The right image shows the SprE-mNeon Green signal after filtering. (b) Population-wide distribution of SprE abundance. The experimental data (blue bars) are shown overlaid with a theoretical Poisson distribution (red dots) of the same average.
1.5 Contributions

Burak Okumus and Charles Baker, Juan Carlos Arias-Castro, Ghee Chuan Lai, Scott Luro and Dirk Landgraf performed the experiments described in this protocol, Charles Baker provided the control and analysis codes and built the system, Somenath Bakshi wrote the spot-detection algorithm, Dirk Landgraf wrote the segmentation algorithm, Emanuele Leoncini designed the growth chamber and wrote the optical density measurement code. Burak Okumus, Charles Baker, Juan Carlos Arias-Castro, Emanuele Leoncini and Johan Paulsson wrote the paper.
It is an old saying, abundantly justified, that where sciences meet there growth occurs.

Sir Frederick Gowland Hopkins

2

Observing Single Cells along the Growth Curve

2.1 Abstract

As microbes deplete local resources and transition from exponential to stationary phase they can change greatly in size, morphology, growth rate, expression dynamics, and intracellular spatial pat-
terns. All these responses can also vary substantially between individual cells, creating phenotypic heterogeneity that in some cases can be advantageous to the population. However, cell-to-cell heterogeneity along the growth curve has so far only been measured at the level of population snapshots, sampling cultures at different time-points but not tracking changes in individual cells. This has made it difficult to determine the progression of events in cells, the stability of various states, the connection between single-cell expression and growth, or the correlation between how cells enter and exit dormancy. To enable quantitative studies of stress response phenomena at the level of dynamics in individual cells, we modified the mother machine set-up for time-lapse imaging of bacteria into a platform for tracking $\geq 10^5$ individual cell lineages in parallel as they change along the growth curve and transition in and out of stationary phase. This required greatly increased throughput, liquid handling for dense and changing cultures, and numerous controls to ensure that the imaged cells closely track the population in a connected batch culture. The final set-up provides a microcosm of bulk growth with unprecedented resolution and control, while making it possible to simultaneously run conventional bulk assays on the same culture. It also provides data for cell physiology and gene expression dynamics for up to 100 million individual cells per day for multiple days in complex growth conditions; enough throughput to study important rare events associated with nutritional stress response, such as persistence to antibiotics. As a proof of principle, we used this platform to study changes in physiology and gene expression in *Escherichia coli* using stress response reporter genes. We then use phase-microscopy to obtain high time resolution (1 min between snapshots) data for the growth of *E. coli* during all phases of growth. We observe the expected adder-mode of growth for *E. coli* in exponential growth, but during entry to stationary phase *E. coli* exhibits a
mixed adder-timer-mode of growth behavior and during exit it exhibits nearly perfect sizer growth behavior. This behavior results in widely distributed cell sizes during stationary phase and rapid return to narrowly distributed sizes upon exit. We observe substantial variability in the divisions carried out by different cells during entry to stationary phase, with some cells dividing three to four times more than others. This variability produces widely distributed lengths in stationary phase, with small cells having performed on average more divisions than large cells. On exit, the reverse is observed with large cells beginning active division before the small cells have divided even once. Surprisingly, when the entire process of entry and exit from stationary phase is tracked, all cell sizes perform effectively the same number of total divisions, yielding no cost to the widely distributed stationary phase lengths. However, after a much longer period of stationarity, small cells are much less likely to exit dormancy in a timely manner than large cells, with none of the smallest 10% of cells dividing even once but over 85% of the largest 10% cells resuming active division. This failure to promptly resume growth upon arrival of nutrients, is a hallmark property of Type I persister cells; cells that our platform is uniquely suited to study. We apply our system to the study of persister formation in a *hipA7* mutant, observing persister formation directly without competition effects impacting the results. We observe an exponential decrease in the number of persisters as the wakeup time before arrival of antibiotics is increased, consistent with a first-order kinetics of exit from dormancy. Unexpectedly, we also observe low levels of growth in the persisters when antibiotics are supplied.
2.2 Introduction

Nearly half of earth’s biomass is thought to consist of prokaryotic cells, more than half of which are estimated to be in a quiescent state [53]. These microbes often exist in environments where resources are limiting, typified by long periods of dormancy punctuated by random arrival of nutrients during which cells must enter a transient period of growth before returning to a dormant, stationary phase once the nutrients are depleted. There is strong selective pressure to resume growth quickly upon arrival of nutrients and continue growth for as long as possible before returning to dormancy. Microbes have evolved complex often heterogeneous responses to these constantly changing environmental conditions. Conventional bulk assays in which microbes are diluted into fresh media then grown to a dense culture naturally capture this transitional growth and its associated response [23]. These bulk assays have been the backbone of much of microbial biology and have uncovered many of the molecular players that mediate the response to starvation [54–57]. However, this process is characterized by heterogeneous responses which can only be observed at the single cell level and are thus invisible to bulk assays [20, 27, 28].

Due to the heterogeneous, dynamic response of microbes to stress, characterizing bacterial stress response along the growth curve necessitates the observation of large numbers of single cells through long periods of growth as well as dormancy. Conventional techniques for imaging single-cells as they grow and divide, such as agar-pads or slide-coverslips, have been invaluable for taking snapshots of a population where the bacteria are imaged shortly after being plated on the agar [24–26]. However, these techniques are not suited to observing bacterial growth. They produce locally heterogeneous
conditions with variable competition for nutrients and there is very little control over the global
environment through time. By comparison, recent developments in microfluidics have made it pos-
sible to track bacterial cells under the microscope through many generations, with tight control of
the local growth environment, uniform conditions, and comparatively high throughput [16, 17].
These microfluidic devices can provide greater statistical power than traditional techniques, allowing
dynamic cellular behavior to be characterized quantitatively [2]. However, previous studies using
microfluidic devices, such as the mother machine, have used simple growth conditions, typically
fresh media. Furthermore, the study of phenomena along the curve often requires higher through-
put than even most current microfluidic devices can provide, since these phenomena are often rare,
for example Type I persisters often occur with a frequency well below 1 in \(10^4\) in stationary phase
[58–60], or occur in a short time window during the transition to stationarity. Thus, developing a
method to track the physiology, growth and gene-expression of single bacterial cells under precisely
controlled transitions between different growth-conditions and with sufficient throughput is of crit-
ical importance to decipher dynamic, heterogeneous, and rare microbial stress-response phenomena,
which would otherwise be missed by bulk methods or studies that lack time-series information.

In this paper, we describe a microfluidics-based platform that allows us to track stress-response dy-
namics in bacterial cells with unprecedented throughput in precisely controlled growth-conditions.
This platform allows us to monitor the physiology and gene-expression of single cells along the
growth-curve as they enter and exit stationary phase multiple times. It can routinely track \(10^5\) sep-
ate cell lineages in parallel, over hundreds of generations, imaging every cell multiple times per
generation with enough resolution for automated segmentation and tracking. This approach can
automatically record the cell properties of $10^8$ cells per day, enabling us to directly observe rare events like appearance and disappearance persister cells, plasmid-loss events and even rare instances of chromosome-loss. With this platform, we can uncover quantitative principles that dictate the dynamics and physiology of cells, as conditions change over time, with single-cell resolution and high-throughput. As a proof of principle, we use this platform to study stress-response in *Escherichia coli* using an *rpoS* transcriptional reporter strain (prpoS-GFP) [61]. We track single lineages of the prpoS reporter strain through two rounds of growth, yielding rich information on the distribution and dynamics of cell lengths, physiology, and fluorescent reporter activity. The dynamic prpoS activity and cell size measured with our growth platform were compared to snapshots of cells taken from the bulk culture. The measurements obtained with our platform agree with the snapshots taken from bulk for both entry and exit from stationary phase, demonstrating that this platform accurately captures the response of cells in bulk culture while also providing single-cell resolution and lineage tracking.

We then apply our growth curve system to probe the growth principles of *E. coli* during entry and exit from stationary phase. Three models for microbial growth are usually considered: the timer model in which the time between divisions is maintained regardless of cell birth size, the sizer model in which division occurs at a fixed volume, and the adder model in which a fixed amount of volume is added during each cell divisions [62]. It has been established that during pure exponential growth *E. coli* length regulation can be well described by the adder model [63], but the model of growth that applies during periods of changing nutrient conditions, such as entry and exit from stationary phase, has yet to be established. Changes in the local environment and suboptimal growing con-
ditions present in traditional imaging media such as agar pads have prevented the study of subtle growth dynamics with the required throughput and precision to accurately distinguish the growth models. However, unlike these conventional methods, our platform can provide both high throughput and uniform conditions during natural nutrient downshifts that occur along a growth curve and upshifts that occur when fresh media is introduced to a stationary culture during exit from stationarity. We use our platform to image thousands of cell lineages with a high-magnification phase objective every minute for more than 24 hours while the cells transition from exponential growth to stationary state and then exit to resume exponential growth again. We show that during entry to stationary phase *E. coli* exhibits a mixture of adder and timer modes of growth, and upon exit from stationary phase it acts as a near perfect sizer.

The observed growth behavior during entry results in broadly distributed stationary phase lengths, with small cells performing more divisions on average than large cells before entering dormancy. When the mean number of divisions is plotted against the stationary phase cell size, binned by percentile, it decreases nearly linearly during entry and increases linearly during exit. For a short stationary phase duration (~8 hours), we find that the divisions during entry and exit offset, so that on average all cells regardless of size do the same number of divisions during the process of entering and then exiting stationary phase. Therefore, for short stationary phase durations, there is no cost to the broad distribution of stationary phase lengths. However, when we observe cells that have been held in stationary phase for one week before re-introduction of nutrients, of the 750 lineages tracked, none of the the smallest 10% of cells resumed growth after re-introduction of nutrients whereas more than 85% of the largest 10% of cells were revived and began actively dividing.
Although typically advantageous, resuming growth quickly may expose cells to threats that specifically impact actively growing and dividing cells, such as many anti-microbial compounds[59]. Thus many microbial populations appear to maintain a very small fraction of cells in the dormant state, a phenomena known as persistence [64]. Our platform is uniquely suited to the study of persistence. Since we can image over 100,000 cell lineages every 5 minutes, we have the throughput required to observe the appearance, growth, and disappearance of large numbers of rare persisters. Furthermore, the design of the mother machine isolates lineages of bacteria in individual trenches, thus unlike bulk cultures, slow or even non-growing cells are not diluted away by faster growing cells. We have used this optimized mother machine to characterize the physiology and dynamics of Type I persisters, which are formed during stationary phase. By providing stationary phase cells with fresh media containing 100 ug/ml of Ampicillin, non-persister cells begin growing and are killed causing them to be washed from the mother machine. After 3 hours, when most cells have been killed, we switch to flowing antibiotic free fresh media into the device for twelve hours. Those cells that remain in the device and begin active division during this twelve hour period are persisters. We find that in a $hipA7$ strain of $E. coli$ persisters are formed at a frequency of 3.6% of the population. If a period of fresh media wakeup is introduced before the antibiotic treatment, there is an exponential decrease in the number of observed persisters as the duration of this fresh media phase is increased. Furthermore, in contrast to previous results, we have determined that persisters exhibit growth during the antibiotic treatment, at a rate 25x lower than that of the exponentially growing population.
2.3 Results

2.3.1 A platform for studying single cells along a growth-curve

We have built a system that can flow dense cultures from a conventional, shaking batch-culture into a highly optimized version of the mother machine microfluidic device. In the mother machine, individual cells grow and divide in narrow trenches fed diffusively by a flow-channel that runs orthogonal to these trenches, Fig. 2.1A-B. Live culture is pumped into these flow channels and as the media is depleted of its nutrients, cells loaded in the mother-machine respond to the changes in the media and undergo the same physiological changes as the cells growing in the culture flask. At any point during the experiment, we can switch from flowing growing culture to flowing fresh media, allowing cells to return to exponential growth after exiting stationary phase. Since the mother-cells are retained in the device for hundreds of generations, the process of switching from flowing culture to flowing fresh media can be repeated multiple times, allowing us to observe the same single-cell lineages as they pass through multiple rounds of stationary phase. The switching process is completely automated, has virtually zero dead-volume and therefore provides near-perfect information about time of switch. The Optical Density (OD) of the culture is monitored continuously using a custom-designed OD-meter, kept in series with the flow path, and therefore we can align any part of the observed single-cell data to the bulk OD. Note that the Optical Density (OD) in this text always refers to OD\textsubscript{600}. The OD-meter allows us to synchronize multiple experiments and correlate specific states of nutrient depletion with their corresponding phenotypes. Unless otherwise specified,
all the experiments described in this paper, are performed in rich-defined medium EZRDM [65], to ensure reproducible growth conditions between experiments. Furthermore, the batch cultures are grown in a baffled flask maintained at 37°C while being actively shaken at 220 rpm, conditions that conform to standard practice for conventional bulk growth. With these conditions in EZRDM, the culture typically reaches an OD$_{600}$ of ~5.3 but in other media we have observed OD$_{600}$ exceeding 10. By growing cells in these conditions, results obtained from our platform can be directly compared to bulk assays, and our platform even allows for such assays to be run in parallel with imaging of single cells experiencing the same batch culture used for the bulk assay. However, unlike fresh media or dilute cultures, the dense cultures produced by these optimized bulk conditions cannot simply be pumped directly into the mother machine.

There are numerous technical challenges that must be addressed to properly flow dense culture from a shaking flask into the mother machine in a way that ensures the response of cells in mother-machine is representative of cells grown in the flask. To ensure the cells are always maintained at a constant 37°C, the batch culture is kept in a custom-built incubator that is connected to the microscope incubator with insulated ducting. This dual-incubator design not only increases modularity, since it is not integrated with a specific microscope, but it also prevents vibration from the shaker being coupled to the microscope, see Supplementary Information. The culture flow path has been optimized to reduce transit time of culture from the baffled flask to the mother machine, thus minimizing the exposure of the culture to conditions outside the culture flask. The flow paths have also been carefully designed to prevent bio-film formation, non-uniform temperature conditions, transmission of bubbles to the mother machine, exposure of cells to surfaces other than inert tub-
ing, and cross-contamination between the fresh-media and culture paths. This includes steps such as removing all T-shaped junctions [66], which can trap cells and nucleate biofilms; adding a calibrated amount of pluronic to the media, to minimize clogging without impacting growth; using only pinch valves, to reduce heat-transfer from the valve to the path; and using custom bubble traps, to serve as OD-meters while also removing the bubbles created by the pluronic in the shaking flask, see Supplementary Information for details.

The mother machine also had to be highly optimized to handle the vast changes in cell size along the growth curve (15-fold in MG1655), the increased throughput requirements of typical growth-curve experiments and to prevent clogging from dense cultures. It had to fulfill these requirements all while maintaining proper diffusive feeding and retention of cells for experiments that typically last multiple days. We have sampled approximately 600 combinations of the width, height, and length of the narrow trenches to find the optimal combination of parameters for each growth-condition. If the trenches are not wide or tall enough, then the cells may not load into the trenches and even if they do load properly, the tight-fit can affect their growth rate. If these dimensions are too large, then the cells can re-arrange themselves or load side by side in a single trench. If trenches are too short, then cells are not retained properly and if they are too long, it can affect the nutrient content at the end of the trench. It is thus important to correctly determine these parameters; however, bacteria often vary substantially in size depending on growth media and environmental conditions, thus no single combination of trench dimensions is optimal for all conditions. Thus, for growth curve experiments we choose trench dimensions that are optimal for exponential phase where cells are largest, namely, trench width (W) of 1.5 μm, trench height of 1.3 μm, and trench
length (L) of 25 μm. We find that this choice of trench size is just tight enough to properly retain
the small stationary phase cells and does not artificially impact growth rates at any point during
the growth curve. Using the correct parameters makes it possible to effectively segment each cell al-
lowing us to measure cell sizes and gene-expression levels of even weakly expressed genes like rpoS,
during entry or exit from the stationary phase.

In addition to optimizing trench dimensions, the layout of the mother machine was altered, the
inter-trench distance was decreased, and the flow channel width was reduced to improve throughput
while still maintaining effective feeding and high image quality. First, we determined the smallest
inter-trench distance that did not introduce significant fluorescent bleed-through artifacts from
point-spread function overlap (Supplementary Information). Next, we redesigned the traditional
mother machine layout so that it had cell trenches on both sides of the flow channel rather than just
one. Next, we reduced the width of the flow-channels, while simultaneously increasing the height
to maintain the same cross-sectional area. The width was chosen so that trenches on the both side
of the channel fit within a single field of view (FOV), 220 μm by 220 μm. In this densely packed
architecture, we can observe 230 individual trenches in every FOV, see Fig. 2.1B.

As a result of this optimization, our platform yields rich, single-cell information about cell size,
morphology, and changes in gene-expression as cells traverse the growth-curve. In a single experi-
ment, we can image 8000 lineages from each of 16 different strains allowing us to track 10^7 lineages
every 5 minutes in parallel over the course of multiple days as they experience multiple rounds of
entry and exit from stationary phase. This allows us to not only to observe rare-events, and mea-
sure accurate distributions of certain properties, but also to correlate gene expression from multiple
Figure 2.1: Accurate high-throughput measurement of cell-growth physiology and gene-expression along the growth-curve. (A) A simplified schematic depicting the growth curve platform. The platform is based on the mother machine microfluidic device (right of the panel) in which cells under observation (shown in red) are grown in trenches while liquid media is pumped through an orthogonal flow channel. To observe growth dynamics, we flow actively growing bulk culture into the mother machine device while continuously observing its Optical Density (OD). As the bulk culture (black cells) flows past the cells in the device (red cells) respond synchronously with the batch culture. At any point, a switch can be made to flowing fresh media, allowing us to observe cells return to optimal exponential growth. The dimensions (W, L, and G in the inset) of the mother machine were highly optimized to meet the demanding requirements associated with flowing dense cultures through the flow channels. (B) The updates to the mother machine design have greatly improved the throughput. We can image up to 16 strains in parallel, with imaging of 500 fields of view (FOV), each containing 230 lineages, in under 5 minutes, giving a throughput of over 100,000 lineages imaged every 5 minutes often for multiple days. Note the range of intensities present in the individual FOVs in the montage of FOVs has been increased for visualization purposes. (C) (TOP) A kymograph showing a single trench of cells expressing a fluorescent rpos transcriptional reporter as it goes through two consecutive rounds of growth as depicted in the panel below. (BOTTOM) Here we show 80 single-cell traces, from a single field of view, of rpos expression and cell size as cells enter and exit from two consecutive rounds of stationary phase. Two cell size traces and two expression traces are highlighted and illustrate high variability between the two rounds of stationary phase. The high-throughput measurements of each property allow us to measure accurate distributions of expression level and cell sizes at any time-point along the growth-curve.
genes at different time-points along the growth-curve, giving us a systems-level picture of the dynamics of a regulon. We can also use this multiplexing setup to flow antibiotics or different stressors at different time-points throughout the experiment.

In this paper, to demonstrate the capabilities of the platform we mainly focus on one gene of interest, the master regulator of the general stress-response in *E. coli*, *rpoS*. The expression-level of *rpoS* is a good indicator for the stress-level in individual cells. We use a transcriptional reporter of *rpoS* (prpoS-GFP) to visualize the magnitude of stress response activation in individual cells, strain SB27. In Fig. 2.1C, we show SB27 cell length, in blue, and *rpoS* transcriptional activity, in orange, for 80 cells from a single FOV, during two consecutive passages through stationary phase.

2.3.2 Dynamics of bacteria in mother machine trenches robustly track the dynamics of cells grown in bulk

Since the goal of this platform is to observe single cells respond to a bacterial growth curve, it is critical that the physiology of cells in the device match that seen in bulk. From the single-cell measurements of cell length over time in our device, we can compute both the doubling rate of cell length per unit time, what we call the natural specific growth rate, and the splitting rate (or inverse of the time between cell divisions). In balanced growth, we measure the growth rate of our reporter strain, and find that the mean specific growth (3.17 ± 0.16 per hour) and the splitting rate (3.15 ± 0.5 per hour) are both equivalent to the bulk growth-rate (3.19 ± 0.2 per hour) measured from the OD of the changing bulk culture. During dormancy, the specific growth rate of the cells is essentially zero (-0.01 ± 0.02 per hour). We attribute the small negative value to the impact of photo-bleaching on
the apparent cell size, i.e. dormant cells do not produce new proteins but existing proteins are con- 
tinually photo-bleached, reducing brightness and thus reducing the segmented area of the cells in 
the image.

Dynamic changes in bulk are captured by the cells in the microfluidic device. We tracked the 
expression of the rpos transcriptional reporter strain (SB27) upon entry to stationary phase. As the 
OD of the batch culture goes through a diauxic shift, the growth rate levels out, going through a 
local minimum Fig. 2.2A. The single cell measurements respond synchronously to this dynamic 
change in the bulk, with the cell length and prpoS reporter fluorescence changing in tune with the 
bulk, Fig. 2.2A. This indicates that the bulk dynamics are impacting cells in the microfluidic device 
with minimal delay and in the expected ways, i.e. cell growth drops and rpoS activity increases. It 
also underscores the power of such a growth curve platform for correlating bulk properties of the 
batch culture with single-cell measurements.

To further ensure that the cells imaged in the mother machine are representative of cells in the 
batch culture, we compared the cell length and reporter activity of the prpoS-GFP reporter strain 
from the device to image snapshots from bulk culture, Fig. 2.2B. For the entry to stationary phase, 
cells were aliquoted from the batch culture at multiple timepoints during growth and imaged un- 
der agar pads. The cells were imaged within 5 minutes of being removed from batch culture and 
care was taken to maintain the cells at 37 °C. The careful analysis of this data is in preparation, but 
a cursory analysis indicates that the dynamics of prpoS activity and cell length match that measured 
in the mother machine of the growth curve platform. Furthermore, an early experiment with a mi- 
crofluidic device called MACS captured a few snapshots of cell length along the growth curve during
entry and was found to agree with the results from the growth curve platform, Fig. 2.2C. The dilute cultures present during exit from stationary phase prevented us from using standard agar pads for imaging without performing perturbative centrifugation. Therefore MACS was used since it is able to concentrate dilute cultures in a field of view for imaging. Similar to what was observed for entry, the results from this control matched that observed with the platform, Fig. 2.2D.

2.3.3 Bacterial growth laws along the growth curve

The ability to watch microbes enter and exit dormancy with single-cell resolution, provides novel information about cell fitness and cell size regulation strategies. During entry to stationary phase, we observe a drop in the specific growth-rate, it then appears to increase briefly, as can be seen in the small uptick in Fig. 2.3A. However, before the specific growth rate can level out, glucose becomes limiting and the specific growth rate steadily declines as the cells prepare to enter stationary phase. The observed dynamics of specific growth-rate are highly responsive to both changes in nutrient quality, as seen during the diauxic shift when the cells change carbon-sources, and changes in nutrient quantity, as seen when cells enter stationary phase where the media has been depleted of nutrients. The splitting rate on the other hand is much more refractory to rapid changes in nutrient quality and availability. It remains essentially constant through the entire diauxic shift heading into stationary phase. The inertia of splitting rates comes from the replication process, as cells can only split into two daughter cells once the initiated rounds of replication are completed. As the replication process takes about 45 minutes to be completed, this means cell splitting takes roughly 70 minutes (45 minutes/ln2) before it is adjusted. Until 70 minutes has elapsed, cells continue to split with
Figure 2.2: Time-lapse phenotyping of single-cells reveals rich information about growth and physiology in complex growth-conditions. (A) The bulk dynamics observed in the flask are synced with the dynamics of the mother machine. When the optical density (black), goes through an inflection point—valley in bulk growth rate (red)—there is a synchronous drop in the cell length (blue) and rpoS transcriptional activity (orange) of the cells in the mother machine device. (B) To determine how well the cells in the mother machine trenches mimic the cells grown in bulk, snapshots were taken of cells grown in the growth chamber flask. Upon entry (C) and exit (D) from stationary phase, the cell length dynamics observed with the growth curve platform are compared to bulk snapshots acquired with MACS. (C) During entry to stationary phase we have plotted the results from the growth curve platform (blue) and the bulk snapshots obtained with MACS (black), where errorbars represent standard deviations. Note that a more thorough control for the entry has recently been completed, with faster sampling and prpoS reporter intensity measurements, and preliminary results show strong agreement with the expected data from the growth curve platform. (D) During exit we take frequent measurements of the prpoS and cell length, plotting the mean values from each image as a single point. This is overlaid on the mean values measured by the growth curve platform for the cell length (solid blue line) and the prpoS reporter activity (solid red line). The standard deviation of the measurements from the growth curve are shown as shaded gray regions.
a constant rate, a phenomenon that is called rate-maintenance, and which has up to this point only been observed in bulk nutrient downshift studies. Observing the rate-maintenance phenomenon in single cells provides a novel insight into how cells regulate their size as they enter dormancy; namely, the rate of specific-growth gradually decreases while the splitting rate remains nearly constant. This causes cells to proliferate in number while decreasing in size during each round of division.

How cells maintain a tightly distributed length, with coefficients of variation (CV) of cell length on the order of 0.1 in many cases, is a fundamental question in the field of microbiology [62]. Three models are typically proposed for how cells control size: adder, sizer and timer. The adder mode of size-control is characterized by the constant addition of length, the sizer is characterized by division at a fixed length threshold, and the timer is characterized by division after a fixed time duration. These three models of size control can be effectively distinguished with a single parameter, $\alpha$, which can be well approximated by $1-C$, where $C$ is the Pearson correlation between cell size at birth ($l_{\text{birth}}$) and division ($l_{\text{div}}$) [62]. In the sizer model $\alpha = 1$, since the size at division is fixed ($l_{\text{div}}$ is constant), thus yielding no correlation between $l_{\text{birth}}$ and $l_{\text{div}}$. In the timer model $\alpha = 0$, since $l_{\text{birth}}$ and $l_{\text{div}}$ are perfectly correlated [62]. The adder represents a compromise between the other two growth modes with $\alpha = 0.5$ giving a strong but not perfect correlation between $l_{\text{birth}}$ and $l_{\text{div}}$ [62]. In the face of the inherent stochasticity in cellular processes, these three models provide differing levels of control over division time and cell size. Although the perfect timer produces the tightest distribution of division times, it is in fact unstable, causing the distribution of lengths to diverge [62]. The perfect sizer produces the broadest distribution of division times but the tightest distribution of lengths [62]. Once again, the adder proves to be a compromise yielding a tighter distribution of sizes than
the timer, though not as tight as the sizer, and a tighter distribution of division times than the sizer but not as tight as the timer.

We used a high-power, phase objective to image thousands of lineages of MG1655 Δ_motA (NDL-93) every minute for over 30 hours to observe growth dynamics during entry and exit from stationary phase. Steady exponential growth is reached roughly 2 hours after the imaging is started and continues steadily for 6 hours. The entry to stationary phase is defined as the one hour period starting when cell length begins to drop, at 8.6 hours, a period during which rate maintenance occurs, The exit is defined for the time period starting at 20.6 hours, when fresh media is added, as indicated by the immediate increase in average length and continuing until 22.8 hours to allow small cells to begin dividing.

As has been previously shown, during periods of pure exponential growth, E. coli exhibits length control consistent with an adder-model (\(\alpha = 0.46\) and \(CV(l_{birth}) = 0.16\)). However, during entry to stationary phase the cells exhibited a mixture of adder and timer (\(\alpha = 0.2\) and \(CV(l_{birth}) = 0.19\)), and during exit from stationarity the cells exhibited near perfect sizer dynamics albeit with a slightly wider distribution of division lengths than expected for such a combination, (\(\alpha = 0.87\) and \(CV(l_{birth}) = 0.15\)). The theoretically derived slopes of the linear fit of division length vs. birth length is two for timer, one for adder and zero for sizer. We observe slopes of 0.998 for exponential phase, 1.23 for entry to stationary phase and 0.06 for exit from stationary phase, consistent with the proposed adder, mixed adder-timer, and sizer models respectively. We also observed that the second division after exit is pure adder, suggesting a rapid return to the control present in exponential growth.
Figure 2.3: Cell size regulation during entry and exit from stationary phase reveals novel control principles. (A) During the entry to stationary phase, the cell splitting rate (magenta line) is constant through the diauxic shift (grey line) but the natural specific growth rate (yellow) drops before the diaxis shift. This indicates that during entry the cells continue dividing without increasing sufficiently rapidly in mass to counteract the division rate. For panels (B–F), we imaged cells with high-magnification, phase microscopy (100x magnification) every minute for more than 24 hours as they grow exponentially, enter and then exit stationary phase. This allows us to extract length data every minute for over 2500 cell lineages. (B) Here we plot 3 sample traces from the cell lineages showing entry into stationary phase. The splitting rate can be seen to remain relatively constant entering into stationary phase, as indicated by the grey lines. (C) We also show three sample traces during exit from stationary phase, illustrating the rapid return to more narrowly distributed lengths at division despite widely differing lengths in stationary phase, indicated by the grey bars. In plots (D–F) we show a scatter (grey dots) of the length at division \( L_{\text{div}} \) and length at birth \( L_{\text{birth}} \) for division intervals during exponential (D), entry to stationary phase (E) and exit from stationary phase (F). The correlation, \( C \), of these scatter points is given on the plot. The theoretical predictions for an adder (red), sizer (purple) and timer (green) are overlaid on the plots. The lengths at division are binned then plotted vs. \( L_{\text{birth}} \) (black circles) and the slopes fitted to these binned points are given on the plots.
As a consequence of the mixed adder-timer growth dynamics during entry to stationary phase, the distribution of stationary phase lengths has a CV two times higher than the CV of birth lengths during exponential growth. The sizer behavior during exit from stationary phase allows these widely distributed stationary phase cell lengths to rapidly return to exponential growth behavior. This large variation in size during stationary phase can also be seen as a difference in the timing with which given cells halt division, with small cells on average going through more divisions than large cells on entry, Fig. 2.4A. Correspondingly, we notice that during short stationary phase durations (8 hours) the large cells begin dividing more quickly than the small cells. When the number of divisions is plotted vs. the stationary phase length, binned by percentile, the points fall on a line with a slope of -1.02 during entry and during exit they fall on a line with slope of 0.85, Fig. 2.4B. In these conditions, the largest 10% of cells do on average $3.14 \pm 0.07$ divisions and the smallest 10% of cells do $3.26 \pm 0.05$ divisions. Therefore, even with the wide distribution of cell lengths in stationary phase, neither the large nor the small cells incur a significant cost in terms of total number of divisions for conditions of starvation that last on the order of 8 hours.

We then used the mother machine to probe the exit dynamics of cells that experience much longer starvation conditions. Cells from a ΔmotA strain of MG1655 (NDL-162) were left in stationary phase for one week, after which they were loaded into the mother machine and provided fresh MBM media. Unlike the eight-hour stationary phase, there was a significant cost to smaller cells for this week long stationary phase. In these conditions, nearly 55% of cells (414 of 750 lineages) did not
begin dividing within 10 hours of introduction of fresh media, Fig. 2.4C-D. The fraction of cells of a given size that eventually began dividing was strongly correlated with stationary phase size, Fig. 2.4D. None smallest 10% of cells woke up and began dividing whereas over 85% of the largest 10% of cells began dividing in that time window.
Cells that are actively dividing become susceptible to external threats, such as antibiotics, that may not impact dormant cells, i.e. persisters. Type I persisters are formed during stationary phase and upon exit from stationary phase the fraction of Type I persisters in the population quickly plummets. This is due to both dilution by the exponentially expanding population of non-persisters and from persister cells randomly switching back into non-persister cells. The dilution effect coupled with the rarity of both persister formation and conversion, make it difficult to observe the conversion process with conventional single cell assays.

We used our platform to measure the fraction of persisters in stationary phase and how frequently persisters switch back to non-persisters after being provided with fresh media. We designed a three-way valve control system that allows us to flow antibiotics into the mother-machine at any time-point during the experiment. After 8 hours of stationary phase, fresh media is pumped into the device for anywhere between zero and five hours followed by a three hour treatment of media with ampicillin (100 ug/ml, 10x MIC). During the ampicillin treatment, the majority of cells are actively growing and are thus killed by the antibiotic, leaving only putative persisters. After the 3 hour antibiotic treatment is finished, we flow antibiotic-free, rich growth media past the remaining, currently dormant, cells and monitor their dynamics for 12 hours to determine which cells return to an actively growing state (Fig 2.4E-F). We applied this assay to a hipA7 persister mutant strain (SJC37).

Given our ability to image more than 100,000 lineages in parallel, we were able to vary the duration of fresh media wakeup between 0 and 5 hours with three replicate lanes per condition. We found that approximately 3.6% of cells were persisters during stationary phase, consistent with previous measurements of ~1% [10]. The frequency of persisters dropped exponentially with time after the
Figure 2.4 (following page): Stationary phase length distribution and the tradeoff between antibiotic resistance and survival of deep stationary phase. (A) Sample of 200 (translucent grey) cell length time-series for (NDL-93) during exponential, entry to stationary, stationary and exit from stationary phases of growth. Two sample traces are shown in blue and red to highlight the differing number of divisions performed during entry and exit for small vs. large stationary phase cells respectively. (B) The number of divisions performed during entry (green), exit (brown) and in total from entry through exit (yellow) are plotted vs. stationary phase cell size binned by percentile. The mean values are plotted with error bars (SEM) and a linear regression line is fit to these points. Although the mean number of divisions during entry and exit is highly correlated with stationary phase cell length, when considered together, the total number of divisions is only very weakly correlated with length, yielding little to no cost to given cell size for this short stationary phase. (C) Cells were subjected to a stationary phase lasting 1 week before being provided with poor media (MBM) for ten hours. Ten sample cell length traces are shown for the largest and smallest 10% of stationary phase cells. The cells that begin dividing are shown in red and those that do not are shown in blue. For the large cells, over 85% of the lineages begin active division, but for the small cells none of the lineages begin active growth. (D) The percentage of cells for a given cell size that began dividing within 10 hours of media replenishment is shown in red and the percent that never began dividing are shown in blue. This demonstrates a potential cost to small cells that is present in long stationary phase durations but not present during short stationary phase durations. (E) Kymograph showing a trench containing a persister mother cell, during a persister exit experiment. The persisters we observed had a distinctive phenotype characterized by high pRNA1-mCherry-mKate2 expression in stationary phase. (F) During such an experiment, fresh media (EZRDM) is provided to stationary phase cells in the mother machine for anywhere between 0-5 hours followed by EZRDM containing 100 ug/mL of ampicillin for an additional three hours. After the ampicillin treatment, the dividing cells have been killed and the remaining dormant cells are recovered in fresh media without antibiotics for 12 hours. Those cells that begin dividing within this time window are persisters. (G) We varied the time of initial antibiotic free fresh media wakeup between 0-5 hours and counted the number of persisters remaining in the device. The frequency of persisters fell exponentially with the duration of fresh media wakeup, consistent with a first order kinetic model for exit dynamics.
cells were re-introduced to fresh-media after being in stationary phase, Fig. 2.4G. Our data is consistent with a first order kinetics of exit dynamics after dormancy. Most persister cells switched to exponentially growing cells within 6 hours of flowing fresh media.

The cells that exhibited persistence towards antibiotic treatment had a characteristic phenotype in stationary phase consisting of short cell length with bright expression of pRNA1-mCherry-mKate2 signal Fig. 2.4E. In contrast to previous findings, the Type I persisters observed in our study are growing even during the antibiotic treatment, albeit at a rate 25 times lower than the mean exponential growth rate. The small increase in cell volume was accompanied by an increase in the expression of pRNA1-mCherry-mKate2 suggesting active gene expression was occurring. This indicates that the observed volume increase was a result of increased protein production, and thus actual growth rather than expansion due to osmotic reasons.

2.4 Discussion

Here we introduced a new platform that allows microbes to be tracked with microscopy as they experience the nutrient starvation of conventional bulk growth, enter into stationary phase and then resume growth upon re-introduction of nutrients. We can image more than 100,000 single cell lineages every five minutes for multiple days, providing a dynamic, detailed picture of the impact of nutrient changes on cell physiology and gene expression. Careful controls were run to ensure the cells in our platform accurately capture the behavior of cells in the conventional bulk flask. Our device achieves ultra high throughput, and can track 6,250 lineages from up to 16 different strains ex-
periencing identical starvation conditions or observe more than 100,000 lineages from a single strain. This allows us to observe millions of division events every day, sufficient to detect rare phenomena such as persister formation.

Using this platform, we determined the growth principles of *E. coli* entering and exiting stationary phase in rich media (EZRDM). When entering stationary phase, small cells divided more times on average than large cells, in some cases performing as many as 3-4 more divisions; this lead to widely distributed stationary phase sizes. We investigated the impact of size on the exit from stationary phase after cells were left in dormancy for short (~8 hours) and long (1 week) periods. Although there was no cost to small size during short sojurns in stationary phase, these small cells were at a significant disadvantage when waking up to poor media after a long period of stationary phase. However, since small cells typically performed more divisions, they could have an advantage in heterogeneous environments, since these cells have additional offspring that can disperse more widely. Furthermore, failing to divide is not always a disadvantage, as it can confer resistance to antibiotics; a phenomenon exhibited by Type I persisters. We examined the formation of Type I persisters in a *hipA7* mutant strain and determined the rate at which they exit from dormancy. In future experiments we plan to look at persister formation in wild-type cells.

We believe that the platform we have described has the potential to uncover many new principles about the dynamics of microbial responses to nutrient depletion and replenishment. It is already being put to use in nearly all projects in our lab, to study phenomena from plasmid loss to ATP production. It is also being used with a variety of microbes such as *B. subtilis* and *Vibrio natriegens* to study basic questions about growth. The time series information provided by our platform can
be used to determine the history dependence of phenomena, e.g. is a specific event triggered by the current culture conditions or instead are the conditions at some point in the past responsible. More generally, it opens to the door to studying hysteresis effects, a subject currently being explored in the lab.

2.5 Methods

2.5.1 Description of growth curve platform

An incubator was constructed using T-slot framing and acrylic, then heated with an OEM resistive heater and fan. The temperature of the incubator is constantly monitored in two places using a custom microcontroller solution and a data-logging thermometer. This incubator houses multiple peristaltic pumps, solenoid valves and a culture shaker. At the beginning of an experiment, a 500 ml baffled flask containing 200 ml of pre-warmed media is inoculated 1:10,000 with liquid culture. It is then immediately placed into the incubator where it is both maintained at 37 °C and actively shaken at 220 rpm. The liquid from a flask containing either fresh media or the actively growing culture is pumped through (mostly) silicone tubing then into the microscope incubator where it is connected to the mother machine with blunt-end needles. In this path, there is a small, sealed chamber that acts to remove bubbles and is also a vessel in which the OD is continuously measured. The custom incubator is connected to the microscope incubator using insulated ducting to ensure that the culture is maintained at a constant temperature throughout the path. Fans also force the heated air through the ducting to ensure the temperature is maintained.
2.5.2 Preparing the growth curve platform for experiments

Before any experiment is run with the platform, an automated system is used to wash the entire media and culture tubing path. The wash consists of an ~40 min wash with 20% bleach, followed by a 40 min wash with 20% ethanol and finally a 40 min wash with dH₂O. Then, just prior to running the experiment a bottle containing EZRDM is hooked up to the platform and pumped through all the tubing to remove the water and replace it with media. While this is taking place, the chip is loaded as described below. Once the fresh media has washed out the water, the media used for wash is removed and the flask containing the growth media is attached to the setup. Blunt end needles are used to connect the loaded mother machine chip to the output of the growth curve platform. At this point, the media is inoculated with a dilution of 1:100,000 and pumped into the mother machine chip.

2.5.3 Mother machine chip preparation

To prepare the PDMS for the mother machine chips, dimethyl siloxane monomer (Sylgard 184) was mixed in a 10:1 ratio with curing agent, defoamed and then poured onto the silicon wafer. This was then degassed for 20 minutes with using vacuum, and then cured at 95°C for 1 hour. Individual mother machine chips were cut out and holes were created for the inlets and outlets using a biopsy punch. Coverslips were cleaned by sonication in KOH followed by sonication in DI water and then bonded to the feature side of the mother machine chips using oxygen plasma treatment (30 seconds at 50 W and O₂ pressure set to 170 mTorr) followed by incubation at 95°C for at least 1 hour. Chips


were bonded the day before being used in experiments.

2.5.4 Cell preparation

EZRDM was prepared from frozen components (Teknova). The media is prepared according to manufactures instructions and then adjusted to obtain optimal osmolarity by adding 15.2ml of 3M NaCl. Media was prepared no more than 4 days before use. Just prior to use in an experiment, pluronic (F108) is added to the media to a final concentration of 0.8%. *Escherichia coli* strains were grown overnight in EZRDM. The cells from this overnight culture cells are loaded into the mother machine chip using gel loading tips and then centrifuged on a custom-machined holder at 5,000g for 10 min to push cells into the side-channels. This is done twice to load both sides of the chip.

2.5.5 Strain construction

BO37 was used in the bulk culture flask. It was built by P1 transducing glmS::PRNAI-mCherry1-11-mKate-T1 terminator-FRT Kan FRT::pstS allele into MG1655 as described in[20].

NDL-93 was a gift from Nathan Lord. A P1 transduction was performed from the Keio collection strain CGSC:9565 [67], motA743::kan into MG1655 and flippase was used to remove the kanamycin resistance.

NDL-162 was a gift from Nathan Lord. It was constructed from NDL-93 by P1 transduction of pRNA1-mCherry-mKate2 Hybrid to a site near attTN7 and flippase was used to remove the kanamycin resistance.
SB27, the prpoS reporter strain, was constructed by P1 transducing $pbo.A$:prpoS_short-GFPmut2-Kan into NDL-162.

SJ[C]37, was constructed by P1 transduction of the hipA7 mutation into NDL-162 and removing the antibiotic resistance marker.

2.5.6 Microscopy and image acquisition

Images used in early experiments (Fig. 1C, 2A,C, 3A and 4C) were obtained with a microscopy setup similar to that described in Potvin-Trottier et. al. [22]. This setup uses a Nikon Ti, microscope equipped with a temperature controlled incubator, an Orca R2 CCD Camera, Nikon 60x Plan Apo oil objective, and a Lumencor LED light source. Microscope control for imaging and control of the Ludl xy-stage was performed using MATLAB interfacing with Micro-Manager. Exposure times were typically between 50-200 ms. Focus was maintained by a combination of an infrared perfect focus system (PFS) and a custom z-stacking routine that is performed every hour to maintain long-term focus. The microscope was equipped with GFP (Semrock GFP-3035B), RFP (Semrock mCherry-A), YFP (Semrock YFP-2427A) and CFP (Semrock CFP-2432A) filter sets.

Images for the other experiments, i.e. the persister analysis, and phase imaging for growth dynamics, were performed on a Nikon Ti2 microscope with an Andor Zyla 4.2 Plus sCMOS camera, Nikon Stage, OKO Labs incubator, and Lumencor LED light source. It has separate emission and excitation filter wheels to increase the speed of imaging. For high-throughput experiments, such as the persister assay, a 40x air objective is used and for the high-resolution phase imaging, a 100x oil phase objective is used. The setup is controlled by the Nikon NIS elements software package.
2.5.7 Image analysis

The image analysis consists mainly of segmentation and was performed using custom scripts in Python, ImageJ and MATLAB. The segmentation uses a combination of local thresholding and adjustable watershed algorithms to detect cell boundaries and create cell masks. These masks are cleaned using binary operations, such as opening and erosions. A filtration is then performed to remove unphysically small or large connected components from the mask. The trenches are detected using DBSCAN on the centroids of the connected components. The mother cells correspond to connected components with centroids at the top of the trench. The putative trench lineages can then be filtered based on the spread of cell mask centroids and number of missing frames.

2.5.8 Data processing: single-cell trace construction

The segmentation algorithm is not perfect, particularly when segmenting phase images, so we define criteria for the phase images to help filter out data that is unphysical. We filter out any division interval where the length at birth over the length at the preceding division differs from 0.5 by more than 10%. We also filter out any birth lengths greater than 6.5 μm and any divisions with lengths greater than 13 μm. For exponential growth, a minimum birth length of 2 μm is chosen. Additionally, there appears to be a slight, ~2 pixel, dilation in the segmentation that is evident when comparing $L_{\text{added}}$ to $L_{\text{birth}}$, since they should be equal, but in our data they differ by a small amount.
2.6 Contributions

Johan Paulsson, Charles Baker, Somenath Bakshi and Emanuele Leoncini developed the platform, designed experiments and interpreted results. Charles Baker, Somenath Bakshi and Emanuele Leoncini designed and built the incubator. Emanuele Leoncini designed and built the OD meters. Charles Baker designed and built the pump control circuitry, valve control circuitry and wash control circuitry. Charles Baker wrote the Python code to control the pumps that runs on a Raspberry Pi with touchscreen interface. Charles Baker wrote the Android application that runs the wash system. Charles Baker wrote the microcontroller code for the pump control, OD measurement, temperature monitoring module, valve control and wash system. Somenath Bakshi and Emanuele Leoncini optimized the fluid flow paths. Emanuele Leoncini wrote the program that measures the Optical Density. Somenath Bakshi and Emanuele Leoncini performed the prpoS double growth curve experiment, deep stationary phase experiment and growth dynamics phase microscopy experiment. Emanuele Leoncini performed the growth curve entry, agar pad control. Charles Baker performed the growth curve exit control. Somenath Bakshi and Silvia Canas Duarte ran the persister experiments. All authors performed data analysis.
2.7 Supplementary Material

2.7.1 Description of the growth curve platform construction and engineering challenges

The core of the platform consists of a custom built incubator made of acrylic and t-slot framing. The inside of the incubator is maintained at 37 °C using OEM resistive heaters with the included PID controller. Custom built temperature and environment loggers record the conditions at several spots in the incubator. Inside the incubator a small shaker maintains a constant shaking at 220 rpm for oxygenation of the media. A baffled flask with no more than 2/5 of the total volume filled with media, critical to achieve a high final optical density, is used to grow the *E. coli*. The culture is pumped out of the custom incubator into the microscope incubator through an insulated duct, with fans at the inlet to ensure the heated air is well circulated inside the duct.

In a standard mother machine experiment where cells are supplied with fresh media, pluronic, a mild detergent, must be added to the media or biofilms will almost inevitably form usually leading to clogging at some point during the experiment. Without pluronic, live culture pumped into the mother machine is even more likely to cause biofilm formation and eventual clogging. Furthermore, even if the device isn’t clogged, biofilms impact the nutrient content of the media, a serious problem when trying to observe the exit from stationary phase. The concentration of pluronic has been controlled to minimize clogging while having no discernible effect on growth rate. However, this additive, coupled with the vigorous shaking of the culture flask, introduces a high density of bubbles
into the media. Unfortunately, when bubbles are pumped into the mother machine, it causes severe oxidative stress and quite often strips the bacteria from the mother machine trenches.

Bubble traps, see Fig. 2.5, were designed to handle the problem of bubbles entering the mother machine and are also used for OD measurement. Liquid entering a container will settle on the bottom, with the air bubbles rising to the top. So, by pumping media into the flask via an inlet at the top, media pumped from the bottom of the flask will be bubble free, Fig. 2.5a. Note this is not possible in the growth flask because the vigorous shaking disperses the bubbles throughout the volume. The simple bubble-trap, Fig. 2.5a, though effective for a while, will eventually result in all media being purged from the flask due to air displacing the liquid. So, in the final design, a modification, which we refer to as the leveler, has been added to prevent the liquid draining out of the bubble trap, Fig. 2.5b. The leveler consists of an inlet inserted into the bubble trap at the desired liquid height, and a pump that continuously pulls fluid, either air or liquid, out of the bubble trap at a high flow rate, Fig. 2.5b. The leveler, has the added benefit of increasing the turnover rate of the liquid in the flask, to the point where the culture in the bubble trap is completely replenished with new culture from the flask in under 90 seconds regardless of the desired rate of flow into the mother machine. This reduces the effective transit time of the culture to the mother machine and thus minimizes the time during which the bacteria experience sub-optimal growth conditions, i.e. in the tubing. Furthermore, each bubble trap contains a small stirbar and a custom built stirring system consisting of a computer fan and magnet. This improves the growth of the cells during the short time until turnover and also prevents the cells from settling to the bottom of the flask.

In addition to observing cells entering stationary phase, we wanted to observe the cells exit sta-
Figure 2.5: Bubble trap designs. (a) The simplest bubble trap consists of an airtight container and a single pump that is used to pull the bubble filled liquid from the culture flask into the container where the liquid settles at the bottom and the air rises to the top. The media that is pumped into the microfluidic device is pulled from near the base of the container and thus is bubble free. However, since the container is sealed, as more bubbles enter the flask, the liquid level will decrease until eventually the entire volume is depleted, rendering the bubble trap non-functional. (b) To prevent the evacuation of the bubble trap, a leveling pump has been added. This leveler actively pumps from the bubble trap back into the culture flask at a very high rate. In this design, the height of the inlet to the leveling path, will determine the height of the liquid in the bubble trap. If the liquid level dips below this height, the leveler will pump air from the flask and if the liquid level is above this line, it will be removed. This design not only prevents liquid loss but it also significantly reduces the time bacteria spend in the bubble trap, thus reducing the effective delay between the bacteria exiting the growth culture flask and entering the microfluidic device.
tionary phase. This consists of simply switching from flowing stationary phase culture into the device to flowing fresh media. To observe the effect of an abrupt switch from culture to fresh media flow, the switch needs to occur physically very close to the mother machine to prevent the mixing of fresh media with culture during transit to the mother machine after switching. This is achieved using a system of pinch valves that not only allow the abrupt switch to fresh media but also to prevent cells left in the culture path from contaminating the fresh media.

Trying to keep fresh media from being contaminated by live culture in the growth platform is a non-trivial task. In addition to needing the correct amount of pluronic, other measures must be taken to avoid contamination. Firstly, it is critical to avoid T-junctions in the flow path, since these tend to trap particles and prevent cells from being effectively flushed out of the path by the arrival of fresh media [66]. Secondly, it was recognized after several failed experiments, that many commercially available valves can trap cells in a similar fashion to T-junctions and this often results in their eventual failure. Furthermore, traditional solenoid valves must be constantly energized to maintain either the open or closed state; this results in valve heating which can be damaging to live culture and overheat fresh media entering the mother machine. Additionally, it is critical to completely separate the culture path and the fresh media path. Even the small amount of bacteria at the junction of a valve can contaminate fresh media after switching. Therefore, a system of five valves must be used to avoid contamination at the junction between the fresh media and live culture paths, Fig. 2.6. Finally, it is important to flow the fresh media at a slightly higher speed at the initiation of the switch to flush all bacteria from any junction or inlet.

Achieving all of these goals has resulted in a system that is substantially more complex than
naively expected and it requires several different control systems to run. A custom built Python in-
terface was designed to control the pumps and runs on a simple Raspberry Pi computer with touch-
screen interface. A wash system was built that allows the entire setup to be automatically cleaned 
before each experiment, as well as in the middle of an experiment if multiple rounds of growth and 
exit dynamics are required. This wash system is controlled by a custom Android program which 
sends Bluetooth commands to Arduino micro-controllers to actuate a series of valves and pumps. 
The optical density measurements are obtained by an Arduino indirectly measuring the current 
induced by a photodiode in response to light transmitted from an IR led through the sample, modi-
ified from [48]. The measurements are regularly calibrated using an off the shelf OD meter to ensure 
consistency and convert analog voltage levels to OD$_{600}$ values.
Figure 2.6: A schematic of the complete growth curve platform. Culture actively grown in a baffled flask, is pumped into an OD meter that also serves as a bubble trap. From the bubble trap, the live culture is actively pumped into a series of pinch valves that direct it to a y-junction manifold that connects to individual inlets in the microfluidic device, normally the mother machine. To observe the exit from stationary phase, it is important to first flush live culture out of the main path. This is done by setting the culture waste valve to waste, shutting the mother machine flow valve, and then rapidly flowing fresh media from the bubble trap to the culture waste valve and out to waste. After flushing out the culture, the culture path isolation valve can be closed ensuring that any tubing in contact with the fresh media path is culture free. The pump speed is then reduced before turning the mother machine flow valve back on. A wash system is connected to the fresh media and culture flasks through a four-way valve that selects whether to flow water, ethanol or bleach. The contents of the flask can be evacuated quickly by opening the blowout valves connected to the high speed leveler pumps.
Plasmids are among the most numerous self-replicators in the biosphere, with many bacteria containing several types of plasmid and each type present in several copies. They can transfer themselves between cells, through conjugation or competence \([31, 68, 69]\), which makes them invaluable tools...
in microbiology and grave concerns for human health, due to their role in the spread of antibiotic resistance [70–72]. However, establishing themselves in a host is only a small part of the plasmid life-cycle. Once inside a new host, the plasmid must ensure that it is propagated to each daughter cell at the end of every cell division without imposing too large of a selective burden on their host. In response to these selective pressures, plasmids have evolved countless clever mechanisms to ensure they aren’t lost from the population, such as toxin anti-toxin systems, active partitioning and multimer resolution systems [73–75]. Specific plasmids may employ all or none of these systems, but every plasmid has at least been found to employ some form of replication control [34, 76–78].

Replication control machinery is central to the function of all plasmids because copy numbers would be dynamically unstable in the absence of control. Specifically, individual replication attempts are always probabilistic to some extent, and plasmids also segregate imperfectly to the two daughter cells at division [29]. Without negative control, a cell that has more than the average number of copies will have more origins for further replication, continuing to produce more copies [29]. Without some restorative force, copy numbers therefore vary increasingly with time, checked only by extinction at low numbers and slowed cell growth at high copy numbers. This is a general problem of systems with self-replication, autocatalysis or positive feedback: fluctuations can be effectively amplified. Because plasmids are strongly affected by fluctuations, they need some form of negative feedback control to counter the fact that each copy must have an origin for self-replication [29].

Many detailed mechanisms for plasmid replication control were identified decades ago [33, 34]. The molecular details of this control vary substantially between different types of plasmids, but most use some type of negative element, like a repressor or antisense RNA, or cis-elements that
titrate away activators such as iterons \cite{34}. When plasmid numbers increase, so do the negative elements, making it harder for each copy to replicate. However, we have more recently found that the kinetic restrictions on how such systems should be designed are extremely strict. First, it is a necessary requirement that any \textit{trans} acting negative elements, like replication inhibitors, are produced in extremely high numbers, to reduce the statistical error in production and ensure that the inhibitor production reflects the plasmid copy number. It is also a requirement that the inhibitors have exceedingly short half-lives, to ensure that the numbers stay up-to-date with changes in plasmid numbers. The inhibitors must further repress replication with very high cooperativity, to ensure that a small change in the plasmid can completely turn on or off new replications. Plasmids must attempt to replicate many times times (e.g. 100 or 1,000 times) for every successful replication, again to ensure that plasmids can quickly recover from dipping to low numbers. Interestingly, every principle we have mathematically identified as an absolute requirement for efficient noise suppression has been found to hold in plasmids. For example, R1 regulates replication using an inhibitor that is produced tens of thousands of times per cell cycle expressed from one of the strongest known natural promoters and has a half-life of around 30 seconds \cite{37}. They achieve high cooperativity, using the CopB system, and they attempt to replicate around 100 times for every successful replication \cite{37}. This makes them very interesting model systems for negative feedback in cells. However, in contrast to the vast amount of molecular information about R1, which rivals that of lambda phage, there are no methods to reliably measure copy numbers in individual cells \cite{38}. Here we first describe the replication control and segregation mechanisms of R1, and review previous attempts to count copies at cell division.
3.1 Plasmid R1

Discovered in the 1960s, plasmid R1 was one of the first plasmids isolated and it remains one of the most studied low copy plasmids [78, 79]. The wild-type plasmid, R1drd19, is a large, 97 kb, plasmid with multiple antibiotic resistance genes, two post-segregational killing systems, Tn21-like transposon insertions and tra genes used for conjugation [80]. Much of this sequence is not needed to stably maintain R1, at least in lab conditions, with over 1/3 of the sequence devoted to the tra operon, 1/4 from a Tn21-like transposon insertion and much of the remaining sequence also not being central to the core replication control. At 97 kb, R1drd19 is exceedingly difficult to manipulate, even with the sophisticated molecular biology techniques currently available. The challenge of working with this plasmid was even more acute several decades ago when the molecular details of R1 replication were being uncovered. This lead to the creation of several versions of the so-called mini-R1, which contains the (approximately) minimal amount of DNA required to stably propagate the R1 plasmid [78]. Although there are countless versions of the mini-R1, all of them contain the the copy number control (cop) system, the origin of replication (oriR) and an antibiotic resistance marker. The standard mini-R1 also has an active partitioning (par) system, but mini-R1 that lacks active partitioning, Par−, is a common mutant. In the following, unless explicitly noted, R1 refers to the mini-R1.
3.1.1 **R1 copy control**

R1 replication is initiated after a sufficient number of RepA proteins, possibly as many as 20, bind to oriR1 making it accessible to the DNA replication machinery \[34, 78, 81, 82\]. It is believed that RepA binds in *cis* with a region denoted CIS, just upstream of oriR1 being critical to mediate this mode of binding \[83\]. Once replication has been started, it proceeds in one direction via theta mode \[34\]. Despite early literature splitting plasmids into classes based on how tightly their replication was coupled to the host replication cycle, so called stringent or relaxed plasmids, follow-up experiments have found that effectively all plasmids, including R1, replicate randomly throughout the cell cycle \[78\]. It has been suggested that this replication may take place at factories in the center of the cells \[84\]. However, this was largely put forth as an explanation for clustering of R1 that was observed in early experiments \[85, 86\], clustering that, for reasons detailed below, we believe may be due to limitations of the methods used in these experiments.

The logic of replication control in R1 is to reduce (increase) the production of RepA when the copy number becomes too high (low). The RepA protein can be translated from two separate transcripts, expressed from the \(p_{RepA}\) and \(p_{CopB}\) promoters. Under normal conditions, RepA is produced primarily by the transcript \(copB\) transcribed from the \(pcopB\) promoter \[78\]. The \(copB\) transcript also encodes the CopB protein, a protein that is normally present at copy numbers of several thousand and which serves primarily to repress \(prepA\) \[78\]. However, when the copy number dips below a certain threshold, \(prepA\) is derepressed allowing the second transcript encoding for RepA, just called \(repA\), to be produced \[78\]. This promoter is approximately 2.5 times stronger than \(pcopB\)
and is thus conventionally believed to act as a rescue system at low copy numbers [87]. Experiments have shown that CopB does not cause plasmid incompatibility and R1 mutants without CopB still control replication [78].

The main mode of replication control in R1 is mediated by the small RNA CopA [78]. CopA is transcribed from one of the strongest known natural promoters, located on the opposite strand of the transcripts encoding RepA. It features two stem loops, one of which binds very tightly to a hairpin loop in the mRNA encoding RepA, a region called CopT that is present on both the copB and repA mRNA [78]. CopA has a half life of less than 1 minute and mutagenesis experiments targeting the hairpin loop region of CopA/T have not found a single sequence with higher binding affinity than the naturally occurring sequence [36, 37, 88–92]. The CopT region overlaps with the RBS for a gene that encodes a small peptide called Tap [93]. The binding of CopA to CopT sequesters that binding site preventing the translation of tap [93]. This in turns inhibits the translation of RepA, since the RBS for RepA is in a stem loop at the 3’ end of the tap mRNA sequence and requires active translation of TAP to relax the hairpin, making the RBS site available. When CopA binds to CopT, it eventually forms a duplex structure which has cut-site for RNaseIII and is eventually cleaved [94]. However, even before the formation of the duplex structure, the CopA/CopT complex adopts four-way helical junction confirmation which is sufficient to inhibit repA translation; thus neither the duplex nor the cleavage are directly involved in the primary inhibition [95].
3.1.2 R1 partitioning system

Plasmid R1 has perhaps the best studied plasmid partitioning system. It is a Type II ParMRC system consisting of an actin-like ‘motor’ protein called ParM which attaches to a DNA adapter protein ParR which binds to a centromere like sequence of DNA known as parC [96]. The parM and parR genes are co-transcribed from the parA promoter flanked by the parC sequence [96]. When ParR binds to the parC region it represses transcription from parA. ParM exhibits dynamic instability, with randomly created nuclei of ParM monomers randomly initiating filament formation and elongation which occasionally enter a state of rapid disassembly [97]. These filaments are stabilized against disassembly by binding to ParR-parC, with full protection from rapid disassembly upon binding at both ends, i.e. to two separate plasmids [97]. It has long been known that this partitioning system greatly stabilizes R1 against plasmid loss and recent experiments by a graduate student in the lab (unpublished) have shown that this stabilization may drop the loss rate as much as two orders of magnitude, from $10^{-4}$ down to $10^{-6}$.

3.2 Challenges to determining copy number distributions

Despite the great biological and medical importance of plasmids, there are currently no published methods for accurately determining plasmid copy number [38]. This is surprising given that the replication and copy number control mechanisms for many notable plasmids have been known for several decades [33, 34]. This makes plasmid replication somewhat unusual in biology, in that the mechanisms are well characterized but the phenomena these mechanisms explain having not been
observed or measured accurately. The reason is that the commonly used methods interfere with natural plasmid function, do not accurately represent plasmid copy number, or both. For example, one might naively expect that putting a constitutively expressed protein on the plasmid and measuring single cell fluorescence would be a good proxy for copy number, reasoning that twice as much fluorescence indicates double the plasmid copy number. However, the plasmids and fluorescent proteins will segregate into daughter cells very differently since after production the fluorescent proteins are not bound to the plasmids. Since the fluorescent proteins are not effectively degraded by *E. coli*, this difference in segregation behavior means the fluorescent protein will not closely track the copy number of the plasmid. This is compounded by noise introduced by gene expression, fluctuating ribosome levels, fluctuating RNA polymerase levels and even fluctuating levels of RNases.

Another method for counting plasmids would be to directly label the plasmid DNA, then count spots. Methods such as FISH, are challenging because of issues with false positives stemming mostly from the number of non-specific interactions far exceeding the number of specific interactions and from the fact that many cells are impermeable to dye [38]. Furthermore, it has been seen that chemical fixation and to an even greater degree permeabilization, can cause spatial rearrangements of intracellular structures [98]. Alternative methods, such as arrays of DNA binding proteins are highly attractive because they work in live cells. However, all previously tested DNA binding arrays were plagued with issues, such as interfering with plasmid partitioning, plasmid replication and causing unnatural plasmid localization [38]. Many of these problems were exacerbated by the tendency of fluorescent proteins to multimerize when placed in close proximity to each other [99].

One method developed by the lab (currently unpublished) is to determine distributions of low
copy plasmids by abruptly halting plasmid replication then counting the number of plasmid containing cells there are in a micro-colony after many rounds of division, Fig. 3.1a. The most sophisticated version of this technique involves cloning arabinose inducible GFP into the plasmid under test, which we refer to as the test-plasmid, Fig. 3.1a. A second plasmid is also cloned that has different replication machinery to the test-plasmid and has the gene for halting replication of the test-plasmid under an IPTG inducible promoter. Cells containing both plasmids are grown in antibiotic free media until early exponential phase, diluted and put onto an agar pad with IPTG, such that individual cells are widely separated. After allowing the cells to grow for several generations, arabinose is added to the media to induce the expression of GFP in cells that still have the plasmid, Fig. 3.1d. Since replication was halted, all cells showing fluorescence should only contain a single plasmid and this plasmid was present in the original cell. Therefore, the number of fluorescent cells equals the plasmid copy number in the founding cell of the microcolony. This assay was subject to an array of validations and tests and appears to work with very high accuracy.
3.3 Developing a fluorescent DNA-tagging based plasmid detection circuit

The replication arrest assay is, to our knowledge, the first assay to determine plasmid distributions without observed artefacts, but it is an incredibly challenging experiment, with low throughput that provides no information about plasmid localization or time dynamics. Ideally, we would want an assay that directly labels DNA in live cells, such as a DNA binding protein fused to a fluorescent protein. However, other members of the lab (Shay Tal and Per Malkus) evaluated a wide range of conventional terO and lacO arrays but found that arrays sufficiently large to create a strong enough signal for detection interfered with natural plasmid replication and/or segregation.

Beyond the biological challenges of producing an artefact-free DNA-binding assay, the task of observing fluorescent spots tends to be technically challenging, with a preponderance of fluorescent spots being observed from multiple sources. In both the mother-machine and MACS, when the devices are observed with a HILO-TIRF microscopy, a large amount of fluorescent 'junk' was quite often found on devices which were newly-made in a clean-room environment. The prevalence and severity of this problem was reduced, but not eliminated, by using more extensive coverslip washing procedures. Additional aberrant fluorescence seemed to be present in the PDMS but the source of this fluorescence is unknown. Furthermore, there appeared to be external sources of fluorescent spots in the media itself, with the fluorescent spots in the background accumulating in the field of view over time. The exact source of all the spots was never fully identified. However, in MACS a fresh sample is pumped into the field of view for each round of imaging, so a simple solution was devised for reducing these issues in MACS experiments, namely, after each round of imaging, a back-
Figure 3.1 (following page): Characterizing R1 plasmid Distributions. (a) The replication arrest assay developed in the lab works by hijacking the native replication control pathway of the test plasmid forcing it to halt replication. Since no new plasmids are produced once replication is halted, after several cell divisions no cell will have more than a single copy of the test plasmid. To determine how many copies of the test plasmid were present in the original cell, GFP on the test plasmid is induced and the number of fluorescent cells are counted. (b) The CRISPR/dCas9 tagging system includes a plasmid expressing dCas9 fused to mNeonGreen, a plasmid expressing the guide RNA and the plasmid being counted. The guide RNA determines which sequence is bound by the dCas9 protein. (c) The MalI tagging system consists of a single MalI protein fused to two copies of mNeonGreen. This protein fusion binds to DNA, including two binding sites in its own promoter, halting its own transcription. This results in bright spots at the location of binding and very little fluorescence in the background. (d) An example microcolony and fluorescent cell observed in a replication arrest assay. (e) Two example cells showing the spots in the control for the CRISPR based tagging, where there is no guide RNA or target RNA. This indicates that this system, in its current form, cannot be used. (f) Two example cells, showing the spots observed for an R1 par+ plasmid tagged with MalI.
Figure 3.1: (continued)

(a) Diagram showing the expression of mNeon proteins under the control of arabinose and IPTG.
(b) Detailed diagram of the protein expression process.
(c) Diagram illustrating the interaction between extra binding sites and the mNeon protein.
(d) Image showing the expression pattern of the mNeon protein.
(e) Image depicting a specific expression pattern.
(f) Image showing another expression pattern.
ground photo-bleaching step was performed with the imaging laser.

3.3.1 CRISPR/dCas9 based DNA tagging

One of the first attempts we made to tag plasmid DNA used CRISPR/dCas9 fused to a fluorescent protein [100, 101], Fig. 3.1b. This technique has been successfully used for tagging DNA in higher organisms but not in *E. coli* [102]. However, unlike active CRISPR systems which will not work in *E. coli*, since *E. coli* cannot repair double stranded breaks in DNA, there is no a-priori reason the dCas9 would be incompatible with *E. coli* [103]. CRISPR can target essentially any sequence that is flanked with an appropriate PAM motif and thus can tag a completely unmodified plasmid of interest. To change the target of the dCas9 protein, only the small guide RNA needs to be modified. However, noise in the expression of CRISPR could impact the reliability of the tagging, and it is also essential to create a strong enough signal from the plasmid-bound dCas9 proteins to overcome the fluorescence of the unbound proteins in the cytoplasm.

Initially, the results from this clone seemed promising since bright spots were visible in the strain with CRISPR/dCas9 based tagging system. However, the controls revealed a major issue. In a strain lacking both the guide RNA and the target plasmid, spots were observed. In fact, despite the dCas9-mNeonGreen fusion being on an IPTG inducible promoter, spots were observed even without induction, presumably due to leaky expression, a problem that was not rectified by switching to an arabinose inducible promoter. It was unclear exactly what caused the false positive bright spots in the control, whether multimerization of the dCas9 forming foci in the cells, or some off target binding effect. However, in our hands this method was not sufficiently accurate to reliably count...
3.3.2 MalI based DNA tagging

Although the CRISPR based method failed to properly detect plasmids, a paper published in 2014 by Hammar et al demonstrated that chromosomes could be accurately counted using a fluorescent protein tagged to the maltose repressor MalI [39]. Unlike classically used DNA binding systems, MalI has binding sites in its own promoter that serve to repress its own transcription [40]. This auto-repression reduces the background fluorescence enough to allow spots to be visualized with as few as two binding sites, compared with the more than 100 binding sites typically used with classic DNA binding proteins such as LacI. When used to label the termini of the chromosome, this method also produced the expected single spot in cells of a size expected to have a single spot, suggesting very small problems with false positives and negatives. This MalI construct was then adapted for plasmid counting. The early results showed promise, with preliminary results for copy number agreeing well with the replication arrest assay.

To improve this system, we varied the type of fluorescent protein, number of fluorescent proteins, number of MalI binding sites, and copy number of the circuit in the hopes of optimizing the signal to noise ratio. The plasmid clones were transformed into a ΔfliC ΔmalI strain with pRNA1-CFP near attTN7 (CB92). All the clones that used either Venus or mNeonGreen showed bright spots of roughly the expected numbers, described below. By contrast, more exotic constructs, such as those using Halo-Tagging or split-FP systems, did not produce distinct foci, presumably due to poor binding. We found that a circuit containing seven total binding sites and MalI fused to two mNeon-
Green fluorescent proteins (MalI-mNeonGreenTandem) produced the best results, with bright, punctate spots visible in cells viewed with MACS and when plated on an agar pads (plasmid pCB23). However, without an alternative means of verification, these fluorescent foci must be interpreted with care.

3.4 R1 copy number

With our optimized clone, we quantified the plasmid copy number for the native mini-R1 plasmid. We performed 11 experimental replicates with the MACS platform, yielding single-molecule resolution images for more than 2500 cells. In those replicates, we found a combined mean copy number of 5.43 with a Coefficient of Variation (CV) of 40%. However, combinations of experiments can increase the apparent CV of the distribution because it convolves minor differences in average copy number due to slight differences in experimental conditions with the true measured CV of the copy number. The largest single experimental dataset for the MalI spot counting assay contained 895 cells, with a mean spot count of 5.40 and CV of 38%, see Fig. 3.2b. By comparison, the replication arrest assay yielded a mean copy number of 6.24, 16% higher than found with the MalI method, and variance of 34%, which is 4% lower than observed with MalI, see Fig. 3.2a. However, due to spot overlap at higher spot densities we expect a slight undercounting from the spot detection method. We also expect possible over-counting from the replication arrest assay due to leaky replication arrest possibly not fully repressing replication during the entire experiment. Despite the discrepancies in the means, which suggest that the average copy number is 6 copies give or take half a copy, both
methods measured very similar copy number distributions. Much of the fluctuations also reflect the asynchronous population of cells, where some cells are much larger than others. Binning the data into size classes suggest that the noise in copy numbers in each size class is closer to 30%. Both methods show sub-Poisson fluctuations, with the replication arrest assay being 5 percentage points lower and the spot counting assay being 4 percentage points lower than a Poisson distribution with corresponding means. We found this rather striking given the self-replicating nature of plasmids, the numerous noisy inputs, and the challenges that feedback control circuits must overcome to function properly in cells. By comparison, we do not know of a single mRNA or protein observed to have distributions that are narrower than Poisson, including those that were engineered or possibly evolved to suppress noise.

Figure 3.2: The detected R1 plasmid distributions are shown overlaid with a Poisson distribution of the same mean, for comparison. The R1 distributions were measured with (a) the replication arrest assay and (b) the MalI spot detection method, respectively.
3.5 R1 plasmid localization

Unlike the previously developed replication arrest assay, the newly developed DNA-binding method provides information about how plasmids are localized in the cell. We determined the location of each fluorescent spot in coordinates of position along the centerline of the major axis and distance from that centerline, using code adopted from oufti [104] to translate from global positions to local length and width positions. The off-axis (width) positions are scaled by the 98th percentile of width to avoid segmentation mask errors skewing the results whereas length was simply scaled by total centerline length. Due to symmetry, all results can be mapped to a single quadrant of the cell. The positions of all the spots detected in the largest R1 dataset are shown as a scatterplot in Fig. 3.3. Overlaid on these positions is a heatmap that shows the density of spots in the local rectangular region. Histograms are also provided, showing the overall density of spots in that width or length slice of the cell, Fig. 3.3.
Using MACS, we observed fluorescent spots distributed throughout the cell with a slight localization near the pole and a weaker localization near the center. This contrasts with previous studies which observed on average two fluorescent foci per cell, a number recognized as far too low for the copy number of R1 [85, 86]. This undercounting was attributed to single spots actually consisting of overlapping signals from clusters of multiple plasmids [85, 86]. It was recognized at the time that this was in conflict with the observation that the segregating unit of R1 appeared to be a single plasm...
mid. Elaborate mechanisms involving replication factories at the center of the cells were devised to explain this phenomena [84]. However, we hypothesize that these foci result from subsequently discovered issues with the methods used for detection, namely FISH and large DNA binding arrays, rather than being of natural biological origin. However, additional controls are planned to ensure that the method we present here properly captures plasmid localization, see below for more details.

As a proxy for likely newborn cells and likely dividing cells, we selected the smallest and largest 33% of cells from the dataset respectively. We observed that the small cells were distributed with a mean of 4.36 and CV of 35.7% whereas the larger cells were distributed with mean 6.49 and CV 33.5%, see Fig. 3.4a. Furthermore, the localization pattern was visualized for these cells and the spots appear to be weakly excluded from the nuclear region. The small cells had fewer spots in the center compared to the regions slightly offset from the poles, as would be expected in a cell with a single nucleoid region. On the other hand, the large cells had fewer spots at the 1/4 and 3/4 positions, as would be expected with two nuclear regions, see Fig. 3.4b-c. These excluded regions become particularly apparent when the histograms showing the fraction of spots binned by length are plotted. Here we see that the fraction of cells at positions in the putative exclusion regions are approximately 1/2 the height of the peaks outside these regions.
3.6 Outstanding questions and future work

The results above suggest that, for the first time, we can start to understand how plasmids behave in individual cells – how they count copies with replication control, how they determine how those copies are partitioned between daughter cells, and what the actual localization pattern of plasmids are in cells. However, our first step will be to further validate the methods. For example, despite the strong agreement with the replication arrest assay, both for averages and the shapes of the distributions, there is always the risk that the observed spots do not correspond to individual plasmid copies. To test this, we plan to incorporate the MalI fusion circuit into the plasmid used for the replication arrest assay. By performing the replication arrest assay with this plasmid, we can test if the fluorescent MalI spots are only found in the cells that express the induced fluorescent protein and that after many generations only one spot is found per cell. This could also provide a tool to study segregation of plasmids at division for cells with only a few plasmid copies, a condition that is relatively rare in the unperturbed cells but very important during the plasmid life cycle. We also plan to directly compare the loss rates of the plasmids with and without MalI DNA binding. Plasmid loss is extremely sensitive to even minor changes in the plasmid copy number distribution. Therefore, even a small impact from the MalI binding arrays on normal plasmid function should reveal itself through quite substantial changes in loss frequency. Using an (unpublished) method developed by a graduate student in the lab, we will be able to visualize the loss rates of these plasmids directly in single cells, giving us detailed information on the impact of the detection circuit on the loss rate of the plasmid. We also want to test the localization properties observed in MACS using agar pads to ensure that the
Figure 3.4 (following page): Localization of spots in small and large cells. (a) Histograms of the spot distributions for the smallest 33% (top) and largest 33% (bottom) of cells are shown overlaid with a Poisson Distribution with the same mean. The small(large) cells have a mean of 4.36 (6.49) spots per cell with a CV of 35.7% (33.5%), and the overlaid Poisson Distribution has the same mean but a CV of 47.9% (39.2%). (b) A scatter plot showing the scaled positions along the length axis and width axis for the smallest 33% of cells is shown. The colorbar indicates how many spots fall within the given rectangular region. A histogram depicting the total fraction of spots along the major axis centerline is shown below. (c) The largest 33% of cells are plotted in an identical manner to (b). It is worth noting that there appears to be evidence of weak nuclear exclusion. In the small cells the spots are more heavily localized slightly offset from the poles, whereas the spots in the large cells are more heavily localized both in the regions slightly offset from the poles and near the center.
Figure 3.4: (continued)

(a) Number of Cells vs. Spots Per Cell

(b) Major Axis Centerline Position vs. Minor Axis Position

(c) Minor Axis Position vs. Major Axis Centerline Position
pressing does not alter the results.

Once the proper controls have been performed, our initial results point to many exciting open questions that can be addressed by our methods. For example, the results from both methods strikingly show that no or virtually no cells that are close to division contain three or fewer copies – despite the fact that the average copy number in that population is about seven per cell. This is much narrower than expected from Poisson distributions. We believe this may be explained by the inducible promoter, \textit{prep.A}. This promoter is not essential for replication control and has typically been viewed as simply a safety net that is only active when R1 dips below approximately 2-3 copies per cell. Such systems could strongly reduce the risk of having very few copies in cells, without substantially affecting the rest of the distribution. To gain insight into the process, we have built reporter strains for the \textit{prep.A} promoter and hope to observe the activation threshold as a function of plasmid copy numbers in single cells. We are also hoping to observe the repressor expressed from this promoter, CopB, though tagging this small protein has proven challenging so far. One question we are particularly curious of is why such a ‘safety net’ would be placed at such a low value, instead of just slightly below the mean copy number. The answer may lie in noisiness of the \textit{prep.A} promoter and the tightness of its activation threshold: if the threshold is placed at low abundances, fluctuations in the threshold will not significantly randomize the copy number.

We also observe many interesting features of the partitioning system. First, the replication arrest method revealed that Par\textsuperscript{+} plasmids appear to follow what is known as the pair-site mechanism, where two copies are sorted actively with high accuracy and the remaining population follow bi-
nomial statistics. This suggests that plasmids pool their resources to a single filament, but it is not known how this is achieved or how the corresponding proteins are regulated in the cell. We expect to gain much more insight into this process by visualizing plasmids after replication arrest, and by using various segregation mutants.

We also hope to investigate another striking observation from the replication arrest method. Specifically, Par\(^{-}\) mutants where all of the partitioning system had been eliminated still displayed partly active segregation at very low numbers. Specifically, for cells with just two copies per cell, the plasmids were significantly more likely to end up in different cells compared to the binomial expectation. For this reason we are particularly interested in getting the MalI system working for Par\(^{-}\) plasmids as well. However, so far we have had limited success: those strains display a substantial drop in the average copy number, in sharp contrast to what is known from bulk methods or the replication arrest method. We are working to resolve this issue by trying different variants of this mutant. In particular, we have recently built another strain where only parts of the partitioning system were knocked out which seems to restore average copy numbers.

3.7 Materials and methods

3.7.1 Strain and plasmid construction

CB92, PRNA1-CFP was inserted into MG1655 in a region near attTn7 by Tn7 insertion. flic was deleted from this background by P1 transduction of flic::KanR from the Keio collection [67]. The MalI region was deleted from this strain by P1 transduction, gift from Johan Elf. After each P1 trans-
duction, the antibiotic resistance marker was removed.

pCB23, was built by isothermal assembly of the replication control circuitry with oriR, parMRC system and chloramphenicol resistance cassette. A dsDNA sequence was ordered for the sequence of five extra MalI binding sites incorporated upstream of the malI promoter. A subsequent isothermal assembly reaction was performed to add the MalI with extra binding sites fused to the mNeon-GreenTandem construct into the plasmid. The entire plasmid has been sequence verified.

3.7.2 Cell handling and preparation

Cells were grown overnight in AB media with 0.1% glucose or 0.2% glycerol with 10% cas-amino acid supplement with appropriate antibiotics. Cells are grown in 10 ml round bottom falcon tubes to ensure that no microscopic debris from re-usable glassware is introduced to the culture. In the morning, the culture is diluted 1:1000 into 2 ml of pre-warmed AB media without antibiotics and allowed to grow with shaking at 220 rpm, 37°C for 2-3 hours before imaging.

3.7.3 Microscopy and imaging

Imaging and microscopy were carried out as previously described [21, 99]. The spot counting was performed using a 514 nm laser and 200-250 ms exposure times typically used to effectively blur out any unbound fluorescent proteins. The system was controlled with ImageJ and software as described in [21]
3.8 Contributions

Johan Paulsson, Shay Tal and Per Malkus designed the replication arrest assay and interpreted results. Per Malkus and Shay Tal built and performed the replication arrest assay experiments. Johan Paulsson, Burak Okumus, Johan Elf and Charles Baker designed the spot counting study and interpreted data. Johan Elf and Burak Okumus built the original MalI fusion for tagging and did the initial analysis of copy numbers (data not shown). Charles Baker and Johan Elf cloned the MalI-mNeonGreenTandem fusion and the Δmali, ΔfliC deletion strain used in the spot counting experiments presented here. Charles Baker performed the copy number counting and localization experiments described in the text. Charles Baker built a modified software platform for segmentation and spot localization based on code provided by Somenath Bakshi.
A.1 Ordering custom components Timing: $\sim 2-5$ d

1. Download the Gerber files for the MOSFET circuit board (contained in Supplementary Data 2), and submit them to a printed circuit board (PCB) manufacturer for construction. We used Seeed Studio, but any alternative PCB manufacturer will do. Be sure to choose FR-4 TG130 board material, two copper layers, 1.6-mm PCB thickness, HASL surface, 0.4-mm-
minimum solder mask dam, 1-oz copper weight, 0.3-mm minimum hole spacing, no blind vias, no half-cut holes and no impedance control. It is important to note that PCB manufacturing can take several days, and shipping may take even longer depending on the location of the PCB manufacturer.

2. Download the laser cutter design files (Supplementary Data 5), and have the design cut using ¼-inch clear acrylic. Note that the designs already take into account the standard cut thickness, 0.01 inch, and the required tolerances are quite loose. Our parts were cut in-house, but any laser-cutting service, such as that offered by Pololu, can be used.

3. (Optional) If you are planning to use the growth chamber, download and extract the laser cutter design files for the vial holder (Supplementary Data 6), and have the top design cut using 0.118-inch black acrylic and the bottom design cut using 0.22-inch black acrylic.

A.2 Assembly of the MOSFET board - Timing: ~2–5 h

4. Solder the LEDs to the circuit board, and clip off the leads. Next, solder the 1-kΩ resistors on the bottom side of the board, being careful not to touch the LEDs with the soldering iron. Solder the N-channel MOSFETs and diodes into place. Finally, solder the screw terminal blocks and DC barrel jacks to the PCB (Supplementary Fig. 1).

Caution: Soldering irons typically operate at >350 °C and can cause serious burns.

Critical step: It is important to solder in this order to avoid difficulty in accessing components with the soldering iron.
Troubleshooting

5. Insert the DC barrel switch into the DC barrel jack, flip the switch to the off position and connect the switch to a 24-V power supply.

6. (Optional) If you are planning to set up cell-sorting functionality, repeat Steps 4 and 5 to make a second MOSFET board.

A.3 Assembly of the digital analog converter circuit - Timing: ~1–3 h

7. Solder the 6-pin male header to the digital analog converter (DAC) board.

8. Connect the two DAC boards to the half-sized breadboard (Fig. 8)

9. Using jumper wires, connect the serial data line (SDA) and serial clock line (SCL) pins of both DAC boards to one of the rails on the periphery of the breadboard, and connect the positive supply voltage (VDD) and ground (GND) of the DAC boards to the rails on the other side of the breadboard. Then wire the A0 pin of one of the DAC boards to the VDD. Next, attach screw terminals, for connecting the pressure regulator, and wire the VOUT pins of the DAC board to the screw terminals (Fig. 8).

A.4 Assembly of the electronic control base - Timing: ~3–6 h

10. Attach four air solenoid valves to a four-valve manifold. Next, attach one air solenoid valve (or two if you are planning to use the growth chamber, or three for the cell-sorting system) to
the inlet side of a second four-valve manifold, and fill the remaining slots with solenoid valve blanks.

11. Screw an M3 to 4-mm barb adaptor into the outlet ports of the air solenoid valves, but not to the blanks.

12. Screw an M5 to 4-mm o.d. push-to-connect adaptor into one of the manifold supply ports, and screw the plug included with the manifold to the other supply port.

13. Attach the air valve manifolds, the MOSFET board, the Arduino Uno and the two pressure regulators to the electronic control base (Supplementary Fig. 2).

14. Use short jumper wires to connect the pins of the Arduino Uno to the corresponding spots on the MOSFET board and the DAC circuit (Fig. 8).

15. Crimp and connect female MATE-N-LOK receptacles to the leads of the air solenoid valves.

16. Cut ~6-inch lengths of stranded 22 AWG wire for connecting the solenoid valves to the MOSFET board (Fig. 8). Crimp and connect male MATE-N-LOK receptacles to each of the pairs of wires. Connect these wires to their corresponding solenoid valves (Fig. 8).

17. Cut two ~10-inch lengths of 4-mm polyurethane tubing, and use this tubing to connect the supply ports of the pressure regulators together with a T-junction. Connect the remaining branch of the T-junction to the filtered, pressurized air.
18. Use two ∼3-inch lengths of 4-mm polyurethane tubing to connect the outlet ports of the pressure regulators to the supply ports of the corresponding solenoid valve manifolds.

A.5 Assembly of the pinch valve base- Timing: ∼3–6 h

19. Connect the three acrylic components of the pinch valve base using no. 4-40 × 3/8-inch-length screws and no. 4-40 × 1-inch-length standoffs, and then attach the pinch valves with no. 2-56 × 3/8-inch L screws (Supplementary Fig. 3).

20. Crimp and connect female MATE-N-LOK receptacles to the leads of the six pinch valves.

21. Measure the distance (D) from the microscope stage, in which the pinch valve base will sit, to the MOSFET board, being sure to leave enough slack to account for stage movements. Cut 12 pieces of 22 AWG stranded wire of length D. Crimp and connect male MATE-N-LOK receptacles to one end of each of the six pairs of wires. Use these wires to connect the pinch valves to the MOSFET board (Fig. 8).

A.6 Assembly of the cleaning reservoir stand- Timing: ∼30 min

22. Assemble the cleaning reservoir stand using four no. 4-40 × 1.5-inch L standoffs (Supplementary Fig. 4).

23. Cut a short piece (∼6 inches) of green PEEK tubing, and attach it to the smaller-diameter inlet on the GL45 VICI-jour cap, using the connector provided with the cap. Connect the
1/4–28 UNF to a 5/32-inch push-to-connect fitting to the other inlet. Screw the cap onto a 100-ml GL45-threaded media bottle.

24. Use 4-mm (5/32 inch) o.d. polyurethane tubing to connect the outlet port of the bleach solenoid valve to the push-to-connect fitting on the bleach reservoir in the cleaning reservoir stand.

25. Repeat Steps 23 and 24 for ethanol and water solenoid valves.

26. Cut an ~6-inch piece of green PEEK tubing, and attach it to the smaller-diameter inlet on the GL45 VICI-jour cap using the connector provided with the cap. Screw the cap onto a 250-ml GL45-threaded media bottle. This will serve as the main waste bottle.

A.6.1 Making the pressure tubes- Timing: ~3 h

27. Using a 0.75-mm diamond drill bit, drill a hole in the cap of a VWR 2-ml screw-cap microcentrifuge tube.

28. Using wire cutters, carefully cut the Luer lock off a 21-gauge blunt-end needle, strip all the adhesive off the needle and insert it through the hole in the microcentrifuge cap. Note that to remove the adhesive, it can sometimes be helpful to place the needles in 70% (vol/vol) ethanol for 20 min.

29. Wrap VWR lab tape around the outside of the cap, as shown in Supplementary Figure 5, being sure to adjust the blunt-end needle so that at least 1/4 inch is exposed above the lip
formed by the tape. Put this to the side for now.

30. Using a 9/64-inch drill bit, drill a hole through the bottom of the 2-ml VWR microcentrifuge tube (Supplementary Fig. 5).

31. Wrap VWR lab tape around the base of the drilled PT, forming a cup. Place the Luer lock side of a 21-gauge blunt-end needle into the cup. Put this to the side for now.

32. Mix 5-min epoxy in a weigh boat.

33. Attach a 14-gauge needle to a 10-ml syringe, and remove the plunger from the syringe.

34. Squeeze the epoxy into the top of the syringe, and replace the barrel carefully.

35. With the needle pointing up, slowly depress the syringe until the epoxy starts to come out of the needle.

36. Use this syringe to dispense epoxy into the tape cups on the cap and base of the VWR centrifuge tubes (Supplementary Fig. 5). It may be necessary to hold the Luer lock needle against the base of the centrifuge tube while the epoxy sets.

   Critical step: Make sure that the epoxy is fully hardened before using the PTs. This can take several hours, even for the 5-min epoxy.

   Pause point: The PTs can be left essentially indefinitely at room temperature.

37. Carry out Steps 27–36 twice, to make a flow layer PT and a control layer PT.
38. (Optional) If you are planning to perform a cell-sorting experiment, repeat Steps 27–36 an additional three times, making a total of five PTs. One of the new PTs, the oil PT, will be used to facilitate collection of captured cells. The other two new PTs will control PDMS valves. One, the capture PT, actuates PDMS valves 1 and 2 to allow flow from the flow layer PT (Fig. 5). The other, the collect PT, actuates PDMS valves 3 and 4 to allow oil to flow into the chip and send the desired cells to the collection outlet.

A.7 Connecting the tubing on the pinch valve base  
Timing: ~30–45 min

39. Snap the two PTs into the PT holder on the pinch valve base (Fig. 9).

40. Connect the silicone pinch valve tubing using barbed Y junctions, attach green PEEK tubing at the outlets of the pinch valves and connect the tubing to the outlet of the flow layer PT (Supplementary Fig. 6). Connecting the pinch valve tubing to the Y junctions can be challenging. Slowly twisting the tubing side to side can help to push it over the barb.

Critical step: It is important to push the tubing fully over the barb to avoid leaks.

41. Measure the distance (L) from the cleaning reservoir stand to the pinch valve base, and cut four pieces of 1/32-inch i.d. silicone tubing of length L. Use this tubing to connect the washing fluid reservoirs and waste bottle to their corresponding pinch valves. Use the short pieces of green PEEK tubing as adaptors between the 1/32-inch i.d. silicone tubing and the pinch valve tubing.
A.8  (Optional) Assembly of the cell-sorting system - Timing: \( \sim 1 \text{ h} \)

Critical: If planning to perform cell sorting or enrichment with MACS, complete the steps below.

42. Attach two solenoids and two blanks to a four-valve manifold base. Then repeat Steps 11 and 12.

43. Use short jumper wires to connect the pins of the Arduino Uno to the corresponding spots on the second MOSFET board as shown in the cell sorting inset of Figure 8.

44. Repeat Steps 15 and 16 for the three air solenoid valves in the cell sorting inset of Figure 8.

45. Bolt the four-valve manifold and assembled MOSFET board to the cell-sorting base in the same manner depicted in Supplementary Figure 2.

46. Screw the 5/32-inch tube to 1/8-inch NPT push-to-connect adaptors to the manual pressure regulator. Then connect the inlet of the four-valve manifold to the manual pressure regulator using the 4-mm polyurethane tubing. This regulator will be maintained at a constant 30 p.s.i. for the entire cell-sorting experiment.

A.9  (Optional) Assembly of the growth chamber upstream of MACS - Timing: \( \sim 4–6 \text{ h} \)

Critical: If planning to incorporate a growth chamber with MACS, complete the steps below.
47. To assemble the top of the acrylic vial holder, insert the LED holders through the holes labeled 'LED' and 'TRANSMISSION', then attach the washer and nut to the side with the engraved text. Next, slot the acrylic pieces together, being sure to include the shelf in the center. Use tape to hold the acrylic pieces tightly together. Finally, use a syringe to dispense acrylic glue into the seams between components, and allow the glue to set per the manufacturer’s instructions (Supplementary Fig. 7).

48. To assemble the base of the vial holder, tap no. 4-40 holes in the acrylic bottom panel. Next, attach a 40-mm computer fan to the base using no. 4-40 × 1–1/4-inch L screws, using 1/8-inch spacers for the top and 3/16-inch spacers for the bottom. Finally, attach a magnetic disk to the top of the fan using double-sided poster tape (Supplementary Fig. 7).

49. Connect the top of the vial holder to the base using 1–3/16-inch spacers and no. 4-40 × 1–1/2-inch screws.

50. Slide the leads of the detector (photodiode) and emitter (LED) into the plastic LED holder inserts. Use 5-min epoxy to glue them in place, and let the glue dry for at least 15 min. Then slide them into the corresponding LED holders on the side of the vial holder (Supplementary Fig. 7).

51. Connect the stirrer (fan) to the 3.3-V power pin on an Arduino Uno. Connect the emitter (LED) and a 47-Ω resistor in series across the 5-V power pin of the Arduino. Connect the detector (photodiode) and 1.5-kΩ resistor in series across the 5-V power pin of the Arduino.
Measure the voltage drop across the 1.5-kΩ resistor using analog pin A4 on the Arduino (Supplementary Fig. 7).

52. Drill a 3/64-inch hole in the periphery of the cap of a 40-ml glass vial. Cut a piece of green PEEK tubing that is ∼4.5-inches long, and push it through the hole until it is nearly touching the bottom of the vial.

53. Following the same procedure as used for the PT (Steps 27–29), insert two 21-gauge blunt-end needles into the cap, and then make a cup around the cap with tape. Fill the cup with epoxy to hold the PEEK tubing and the blunt-end needles in place. Let the epoxy harden fully.

54. Attach a normally closed pinch valve to the external fill slot on the pinch valve base with two no. 2-56 × 3/8 inch screws. Insert a Y junction at the inlet to the master pinch valve, and connect it to the pinch valve tubing. Insert an ∼1-inch piece of green PEEK tubing into the free end of the pinch valve tubing.

55. Use 1/32-inch i.d. silicone tubing to connect the PEEK tubing on the growth chamber cap to the external fill pinch valve.

56. Connect the tubing from a normally open pinch valve to one of the needle inlets of the growth chamber; this will allow passive aeration when the culture is not actively being pumped out of the growth chamber.

57. Cut an ∼3-inch piece of 4-mm polyurethane tubing, connect a male Luer lock to 3/32-inch
barb fitting to one end and attach the other end to the outlet of the growth chamber solenoid valve. Connect a 21-gauge blunt needle to the Luer lock fitting. Use Tygon tubing to connect this needle to the final inlet to the growth chamber.

58. The two pinch valves and the corresponding air solenoid valve are controlled by a single Arduino pin via the MOSFET board. Connect the leads of the pinch valve and solenoid valve together, and then wire them to the remaining screw terminal slots on the MOSFET board (Fig. 5).

A.10 Ordering the masks - Timing: ~1–3 d

59. Download the provided AutoCAD files (Supplementary Data 1) for mask designs, and submit them to a printing service to obtain transparency masks for the control and the flow layers. Use the maximum resolution possible for printing (20,000 dpi or higher). They should be printed emulsion side down such that the features are on the bottom when the masks are in the correct orientation. The masks have negative polarity, so the features should be clear. Before shipping, ask for a PDF proof for review. We order our masks at CAD/Art Services, but suppliers such as Advance Reproduction, Artnet Pro or Infinite Graphics are good alternatives. Typically, the commercial vendor will take several days to send the transparency masks.
A.11  Cleaning the silicon wafers - Timing: ~30 min

60. Set up the spin-coater to run two consecutive stages. Configure stage one for spreading with angular frequency: 500 r.p.m., ramp: 100 r.p.m./s, time: 15 s. Configure stage two for spin-coating with angular frequency: 3,000 r.p.m., ramp: 500 r.p.m./s, time: 45 s. These are the default settings, unless stated otherwise.

61. Place a silicon wafer on the spin-coater with the reflective side up. To clean the wafer, run the spin-coater, and during the second stage, generously squirt acetone onto the wafer. Repeat this process with methanol and then isopropanol.

Caution: Methanol is highly toxic; upon accidental ingestion, it can cause blindness or death. Wear appropriate laboratory clothing when handling it, and use it in a fume hood.

62. Place the clean silicon wafer with the shiny side up on a hot plate at 95 °C for 5 min.

A.12  Master for the control layer - Timing: ~1 h

63. Place a cleaned silicon wafer on the spin-coater.

64. Pour enough SU-8 2025 over the silicon wafer to cover half the surface.

Caution: Photoresists are highly toxic. Wear appropriate laboratory clothing when handling them, and use them in a fume hood.

65. Run the spin-coater according to the configuration in Step 60.
66. Pre-exposure bake. Place the SU-8-coated silicon wafer with the SU-8 side up on a hot plate at 65 °C for 1 min, and then transfer the wafer to another hot plate at 95 °C for 5 min.

67. Cool the wafer to room temperature.

68. Using the mask aligner, align the control layer mask to the coated silicon wafer.

69. Expose the wafer on the mask aligner to a total dose of 150–160 mJ/cm² at 405-nm UV light (e.g., expose the wafer for 6 s using a 25 mW/cm² mask aligner power density).

70. Post-exposure bake. Place the wafer with the feature side up on a hot plate at 65 °C for 1 min, and then transfer the wafer to another hot plate at 95 °C for 5 min.

71. Cool the wafer to room temperature.

72. Develop the wafer using SU-8 developer until the features appear (~5 min).

Caution: Photoresist developers are highly toxic and reactive. Wear appropriate laboratory clothing when handling them, and use them in a fume hood.

Troubleshooting

73. Rinse the wafer with isopropanol until the channel design is clearly visible. The control layer master is now formed.

74. Hard bake. Place the master, feature side up, on a hot plate at 150 °C for 5 min.

Pause point: Hard-baked masters can be stored at room temperature in a dust-free environment essentially indefinitely.
A.13 Master for the flow layer - Timing: ~1 h plus 8 h of hard baking

75. Place a clean silicon wafer on the spin coater.

76. Pour enough MCC primer 80/20 onto the silicon wafer to cover half the surface, and run the spin-coater according to the configuration in Step 60.

Caution: This reagent is highly toxic and reactive. Wear appropriate laboratory clothing when handling it, and use it in a fume hood to prevent inhalation.

77. Pour enough AZ 10×T onto the silicon wafer to cover half the surface, and run the spin-coater according to the configuration in Step 60.

Caution: Photoresists are highly toxic. Wear appropriate laboratory clothing when handling them, and use them in a fume hood.

78. Pre-exposure bake. Place the silicon wafer with the coated side up on a hot plate at 110 °C for 3 min.

79. Cool the wafer to room temperature.

80. Align the flow layer mask to the coated silicon wafer using the mask aligner.

81. Expose the wafer on the mask aligner to a total dose of 1,425 mJ/cm² at 405 nm of UV light (e.g., expose the wafer for 57 s using a 25 mW/cm² mask aligner power density).

82. Develop the master for 5 min in a 4:1 solution of water/AZ 400K developer (vol/vol).
Caution: Photoresist developers are highly toxic and reactive. Wear appropriate laboratory clothing when handling them, and use them in a fume hood.

Troubleshooting

83. Rinse the wafer with water until the channel designs are clearly visible. The master is now formed.

84. Reflow. Place the master with feature side up on a hot plate at 150 °C for 5 min.

85. Hard-bake the master overnight by placing it feature side up on a hot plate at 260 °C.
   
   Pause point: Hard-baked masters can be stored at room temperature in a dust-free environment essentially indefinitely.

A.14 Making the PDMS control layer - Timing: ~1 h

86. Make a circular aluminum foil cup for the control master, using the base of a 10-cm-diameter Petri dish as a mold. To support the fragile base of the aluminum foil cup, place it in the lid of the Petri dish.

87. Place the control layer master in the aluminum foil cup.

88. Combine 42.5 g of PDMS monomer with 8.5 g of curing agent (5:1 PDMS/curing agent ratio (wt/wt)). Using the conditioning mixer, mix for 2 min at 1,000 r.p.m., and degas for 2 min at 800 r.p.m.
89. Pour 35 g of the 5:1 PDMS/curing agent mixture over the master. At the same time, use a
cotton swab to gently press the master against the bottom of the cup to prevent the PDMS
from leaking underneath the master. This makes ∼5-mm-thick chips.

90. Place the PDMS mixture in a desiccator chamber until all the bubbles have been removed (~
30 min).

Troubleshooting

A.15 Making the PDMS flow layer 2 - Timing: ~30 min

91. Place the flow layer master on a spin-coater. Change the angular frequency in the centrifuga-
tion setup of the second stage in Step 60 to 1,250 r.p.m.

92. Combine PDMS and curing agent in a 20:1 (wt/wt) PDMS/curing agent ratio (for a total
of 25 g), and then mix as described in Step 88. Pour enough of the mixture onto the center
of the master to cover half of its surface. Run the spin-coater. This makes an ∼65-μm-thick
PDMS coating.

93. Place the master in a plastic Petri dish with the PDMS-coated side facing up.

A.16 Thermal bonding of the control and the flow layers - Timing: ~5–14 h

94. Place both masters in a drying oven at 65 °C for 33 min, and then move them to room temper-
ature.
Critical step: This step is time-sensitive. If left in the oven for >33 min, the PDMS will cure completely, and the layers will not bond.

95. Carefully peel the PDMS off the control layer master.

96. Cut out the chips using a single-edge razor blade.

97. Using a 0.75-mm biopsy punch, make holes through the valve inlets (Fig. 1b) on the control layer.

98. Carefully adhere a strip of Scotch Magic Tape to the cast side of the PDMS chips, and then remove the tape in order to remove any fibers that might have fallen onto this face of the chip.

99. Place the PDMS control layer slabs on top of the PDMS-covered flow layer master, being careful to align the features on the two layers. Namely, the channels of the control layer must be perpendicular to the channels of the flow layer.

Critical step: If bubbles form between the layers, try to remove them by gently tapping the PDMS with tweezers. If bubbles persist, the control layer slab can be gently removed and reset on top of the flow layer, as the bonding at this stage is reversible. However, this is time-sensitive and should be done immediately.

100. Place the Petri dish holding the flow layer master in a drying oven at 65 °C for 4 h.

101. Using a new razor, cut out the bonded, two-layer PDMS chips, and carefully peel the chips off the flow layer master.
Critical step: Use a new razor blade, as a dull blade can tear the thin spin-coated PDMS layer.

102. Using a 0.75-mm biopsy punch, make holes through the inlets and outlets of the flow channels (Fig. 1b).

Critical step: Do not punch through the holes made in Step 97, as they are meant to go through only the control layer.

103. Submerge the PDMS chips in a beaker of isopropanol, and sonicate them for 45 min at room temperature.

104. Remove the PDMS chips from isopropanol, rinse them with Milli-Q water, submerge them in a beaker of Milli-Q water and sonicate them for 45 min.

105. Use pressurized air to blow any excess liquid from the PDMS chips.

106. Place the PDMS chips in a covered Petri dish, and dry them in an oven at 65 °C for 4 h.

Pause point: The chips can be stored in the Petri dish at room temperature for several days.

A.17 Preparation of the cover glass for plasma bonding - Timing: ~2 h

107. Place several cover glasses in a beaker of 1M KOH, and sonicate them for 30 min.

Caution: KOH can cause severe burns and eye damage, and is harmful if swallowed. Wear gloves and personal protective equipment and dispose of as hazardous waste.

108. Remove the cover glasses from the KOH, and rinse them with Milli-Q water. Then submerge them in a beaker of Milli-Q water, and sonicate them for 30 min.
109. Remove the cover glasses from the water, and blow them with clean pressurized air to remove excess water.

110. Place the cover glasses in a drying oven at 65 °C for 15 min.

Pause point: Cleaned cover glasses can be stored at room temperature in a dust-free environment essentially indefinitely.

A.18 Plasma bonding - Timing: ~2 h

111. Configure the plasma cleaner by performing a mock plasma treatment with an empty plasma chamber. Turn on the vacuum, flow oxygen into the chamber, wait for ~2 min to allow the pressure to stabilize and then adjust the chamber pressure to 200 mTorr. Set the plasma power to 100 W, wait 11 s, and then turn off the plasma power.

112. Place several clean cover glasses and PDMS chips with the feature sides up, in the plasma cleaner, and run a cleaning cycle with the above settings.

113. Immediately after removing them from the plasma cleaner, carefully place one of the cover glasses, plasma-treated side down, on top of the feature side of one of the PDMS chips. If needed, tap gently on the cover glass to remove air bubbles. Do not apply pressure, as this can cause the collapse of flow channels and hence their irreversible closure. Repeat this step until all PDMS chips have been bonded.

Critical step: Binding between the cover glass and PDMS happens very quickly and is practically irreversible. So make sure to carefully center the cover glass over the PDMS chip, as the
cover glass cannot be repositioned after contact with the PDMS.

114. Place the chips, PDMS side up, on a hot plate at 150 °C for 5 min.

115. Put the bonded MACS chips in a covered Petri dish, and place the dish in a drying oven at 65 °C. Unless you are planning to use the chips for single-molecule imaging, remove the chips from the oven after 1 h, and leave them at room temperature for 24 h before using them. For single-molecule imaging, leave the chips in the oven for 3 d to allow the PDMS to stiffen.
Pause point: The bonded MACS chips can be stored in a Petri dish at room temperature for many weeks. However, the PDMS does slowly cure over time, so chips stored for extended periods tend to be stiffer.

A.19 Connecting tubing to the PTs and the PDMS chip - Timing: ~30 min

116. Place the pinch valve base on the microscope stage. Put the PTs in the holders on the pinch valve base.

117. Cut an ~3-inch piece of 4-mm polyurethane tubing, connect a male Luer lock to 3/32-inch barb fitting to one end and attach the other end to the outlet of the flow layer solenoid valve. Connect a 21-gauge blunt needle to the Luer lock fitting.

118. Measure the distance (D) from the flow layer solenoid valve to the flow layer PT. Cut a piece of Tygon tubing of length D, attach one end to the blunt-end needle at the outlet of the flow layer solenoid valve, and attach the other to the inlet on the cap of the flow layer PT (Fig. 9).
119. Repeat Steps 117 and 118 for the control layer PT. If you are planning to perform cell sorting, repeat Steps 117 and 118 an additional three times, connecting the oil, capture and collection PTs.

120. Using wire cutters, carefully cut the Luer lock off a 21-gauge blunt-end needle. Bend the needle 90°. Prepare three 90°-bent needles in total (or eight if you are planning to do cell sorting).

121. Cut an ~5-inch piece of Tygon tubing, insert one of the 90°-bent needles into one end of the tubing, and connect the other end of the tubing to the outlet of the ‘to chip’ pinch valve. Carefully insert the bent needle into the flow layer inlet, keeping the microscope slide flush against a clean, flat, hard surface to prevent it from cracking (Fig. 9).

122. Cut an ~3-inch piece of Tygon tubing. Insert one of the 90°-bent needles into one end of the tubing, and connect the other end to the outlet of the control layer pressure tube (Fig. 9).

123. Fill the control layer PT with Milli-Q water. Wait for gravity to completely fill the tubing at the outlet of the control layer PT, at which point the water will be dripping slowly out of the blunt-end needle. Carefully attach this blunt-end needle to the control layer inlet (Fig. 9).

124. Cut an ~10-inch-long piece of Tygon tubing for the flow layer outlet (waste). Insert a 90°-bent needle into one end of the tubing and insert the other end into the 40-ml MACS waste bottle. Gently insert the blunt-end needle into the flow layer outlet (waste) (Fig. 9). If you are planning to conduct a cell-sorting experiment, repeat this for the collection outlet.
A.20  (Optional) Connecting tubing to the cell-sorting chip - Timing: ~10 min

Critical: If planning to perform cell sorting or enrichment with MACS, complete the steps below.

125. Connect the outlet of the capture tube to PDMS valves 1 and 2 (Fig. 6). To do this, attach ~2 inches of microflow tubing to the outlet of the capture PT. Connect the other end of the microflow tubing to a barbed Y-junction, and attach two ~3-inch pieces of microflow tubing to the remaining barbs. Finally, insert 90°-bent needles into the two 3-inch pieces of microflow tubing, and carefully insert the needles into the inlets for valves 1 and 2 (Fig. 6).

126. Repeat Step 125 to connect the collect PT to PDMS valves 3 and 4 (Fig. 6).

127. Cut an ~5-inch piece of Tygon tubing, and insert one of the 90°-bent needles into one end of the tubing. Attach the other end of the tubing to the outlet of the oil PT. Then gently insert the needle into the oil inlet of the chip.

128. Fill the oil PT with droplet generation oil.

A.21  Running MACS - Timing: ~2–8 h

129. Place the MACS chip on the microscope stage. If you are using a nonadjustable stage holder that accepts only 75 mm × 25 mm cover slides, the MACS chip can be mounted using the acrylic adaptor provided. Download the Adobe Illustrator file (Supplementary Data 7), and
have the adaptor cut in 1/16-inch-thick acrylic with a laser cutter; see Step 2 for details on laser cutting.

130. Start Micro-Manager, and open the Macs Controller plug-in. In the prompt of the pop-up window, choose 'MACS_AND_CLEANING' for a standard MACS experiment (Steps 131–140), 'CELL_CAPTURE' for a cell-sorting experiment (following Steps 131–146) or 'GROWTH' for an experiment using the growth chamber (as detailed in Steps 147–162).

131. Select the 'Direct Control' tab, set the 'Control Layer Pressure (psi)' to 20 p.s.i. and the 'MACS Device State' to 'Closed' and then click 'Apply'. Wait until the control layer has been filled with water (AKA dead-end filling), which may take up to 15 min. The filling will be evident by a change in the contrast of the control channel.

Critical step: Watch out for possible delamination of the chip. If the two PDMS layers are poorly bonded, this will cause all the water to leak rapidly from the edge of the chip. If this happens, the chip must be replaced.

132. Optional) If you are planning an automated acquisition, open the multidimensional acquisition software in Micro-Manager, select the parameters for your acquisition and save the acquisition settings in an XML file.

133. Engage the 'Live View' in Micro-Manager, and use bright field to focus on the position in which the flow layer channel being used intersects with the control layer channel being used (Fig. 6d).
134. Select the parameters for your configuration in the 'Run MACS' tab. For example, in a typical experiment, we set the 'Flow Layer Pressure (psi)', 'Control Layer Pressure (psi)' and 'Imaging Pressure (psi)' to 20 p.s.i., then we specify the duration of the open state as 5 s, the duration of the half-open state as 3 s and the duration of the closed state as 6 s. By default, the system is configured to spend 2 s in the imaging state before finishing, but this can be adjusted using the properties file described in Equipment Setup.

135. Clean the MACS system as described in Box 1.

136. Unless you are using an integrated growth chamber, remove the cap, and pipette 600 μl of your sample that contains the desired OD of cells (e.g., *E. coli* expressing SprE-mNeonGreen for single-molecule counting experiments) into the PT of a previously cleaned MACS setup.

137. Begin by clicking the 'Inject Sample' button, which will flow the sample from the PT into the MACS chip at the specified Flow Layer Pressure (typically 20 p.s.i.) for the specified 'Cell Injection Duration (sec)'. The injection time is largely determined by the length of the tubing from the to chip pinch valve to the inlet of the flow layer; for our setup, this distance is ~6 inches and the injection time typically used is 60 s. The injection has run long enough if the cells can be seen flowing past the 'Live View' window.

138. While observing the field of view in the 'Live View' window, click the 'Run MACS' button. If the parameters are correct for the given experiment, then the cells will be seen flowing past the field of view while in the open state; they will accumulate in the half-open state (Fig.
5a); they will be trapped in the closed state; and finally they will be pressed with 'Imaging Pressure'; typically, all this happens in \(\sim 15\) s, but timing will depend on the timings specified in the program.

Troubleshooting

139. Once the cells have been loaded and the parameters have been optimized, select the acquisition file (XML) from Step 132 by pressing 'Choose File' under the 'Acquisition' pane. Once a file has been selected, the 'Run Acquisition' button will become enabled. Press 'Run Acquisition' to begin capturing snapshots of the cells with MACS. After acquisition, cells of interest can be sorted according to (optional) Steps 141–146.

140. (Optional) Clean the MACS system as described in Box 1.

A.22 (Optional) Cell sorting with MACS - Timing: \(\sim 4–8\) h

141. After Step 139, switch to the 'Direct control' tab, and set the 'Flow Layer Pressure (psi)' and 'Valve pressure (psi)' to the values that achieve the half-open state, as determined in Step 138. Make sure that the 'Macs Device State' is set to 'Resting' and then click 'Apply'.

142. Switch to the 'Cell Sorting' tab, and click the 'Half-Open' button to start the flow of cells past the field of view.

143. Watch the cells flowing past and, as soon as the cell of interest is spotted in the field of view, immediately press the 'Catch Cells' button. This will halt the flow of cells past the field of
view, and close all PDMS valves, except the control valve.

144. To collect the cells of interest, press ’Collect Cells’ and wait until the cells of interest flow into the collection tube. This may take a few minutes.

145. Repeat Steps 142–144 for as many samples as desired.

146. Add fresh growth media to the captured cells, and allow them to grow overnight.

A.23 (Optional) Running a growth chamber experiment with MACS - Timing:

~2–18 H

147. Download and extract the files in Supplementary Data 8.

148. Open the Arduino IDE, and upload od_measurement.ino, from Supplementary Data 8, to an Arduino Uno.

149. Open MATLAB, and change the directories to the location of the MACS growth curve code, extracted in Step 147.

150. Run growth_chamber_macs.m. Note that all output files from this program will be saved to an output directory created on the desktop by default.

151. In the first window that pops up, enter the strain name and any desired notes into the corresponding fields and then click ’next’. This information will be saved to a text file in the output directory.
152. In the second window that pops up, click the 'Single Measure' button, and wait for the 'Transmission value' (T) and 'OD value' (OD) to be updated (this takes ~10 s). Ensure that the photodetectors are responding to variations in incident light by manually blocking the opening between the IR LED and the photodetector.

153. If a calibration has not been performed for the given combination of growth media and cells of interest, or if the growth chamber has been idle for longer than ~1 month, calibrate the system as described in Box 3.

154. Add 35 ml of growth media and a sterile magnetic stir bar to the sterile growth chamber vial. Make sure that the stirrer is spinning. If not, it may be necessary to give the fan a slight push to break static friction and initiate rotation.

155. Provide the maximum transmission value expected during the experiment by pressing 'Measure to Max'; this will enter the most recently measured transmission value into the box next to 'MaxT'. This value will be used to properly scale the figure axes for the experiment. It does not affect the measurements or output.

156. Enter the desired measurement interval, in seconds, into the 'Time btw measurements' panel. The measurement intervals should be longer than 2 s. We typically use 20 s for this interval.

157. Dilute an overnight culture in the growth media; we typically perform a 1:100,000 dilution to observe a sustained balanced growth.

158. To begin timed measurements, click the 'Start' button. The first measurement will take ~10–
15 s to appear on the OD plot. All proceeding measurements will take place after the specified measurement interval.

159. Clean the MACS system as described in Box 1.

Critical step: Make sure that the durations for the active and passive filling of the PTs by the cleaning fluids, as described in Box 1, step 3 are entered correctly before proceeding. These will be used to clean the system between rounds of acquisition, if selected.

160. Inject the culture into the MACS PT by clicking the 'Actively Fill PT from GC (sec)' button on the 'Run Macs' tab of the Macs Controller plug-in.


162. (Optional) A process of running repeated acquisitions, with fresh samples being taken from the growth chamber each time, can be configured in the 'Run Macs' tab. Simply, choose whether the flow PT should be cleaned after every acquisition, the number of acquisitions to run and the time before each new acquisition. Click 'Choose File', and then select the acquisition settings file created in Step 132. Then click 'Run Acquisition' to begin imaging.
References


