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Microfluidics and single-cell microscopy to study stochastic processes in bacteria

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Abstract

Bacteria have molecules present in low and fluctuating numbers that randomize cell behaviors. Understanding these stochastic processes and their impact on cells has, until recently, been limited by the lack of single-cell measurement methods. Here, we review developments in microfluidics that enable following individual cells over long periods of time under precisely controlled conditions, and counting individual molecules in many cells. We showcase discoveries that were made possible using these devices in various aspects of microbiology, such as antibiotic tolerance/persistence, cell-size control, cell-fate determination, DNA damage response, and synthetic biology.

Introduction

Bacterial cells contain many mRNAs, proteins, and other components in few and varying numbers per cell. Because the levels of one component affect the reaction rates for making or degrading other components, this heterogeneity can spread through interaction networks and affect any aspect of cellular life. From first principles, substantial heterogeneity may thus be expected in most bacterial processes unless there is a strong selective pressure to minimize it, which tends to be mechanistically difficult and energetically expensive [1]. Indeed such heterogeneity has been observed in virtually every process studied, from mutation rates [2] to gene expression [3–6] to cell growth and division [7]. For these studies, snapshots of individual cells are much more informative than bulk averages, since the width and shape of the distributions reflect the kinetics of underlying reaction processes (Figure 1a). However, a great deal more information can be gained from time-series data (Figure 1b), e.g. whether the heterogeneity slowly meanders up and down or changes in sudden steps.

The development of more reliable probes and faster imaging technologies in recent years has led to an explosion of image-based analyses of dynamic processes in bacteria. For time-lapse imaging of bacteria, the most common approach has been to seed cells on agar pads and track the growing micro-colonies over time. While such conditions are often no less

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representative of natural habitats than those of microfluidic environments, the lower throughput, shorter time series, and greater difficulty tracking lineages of growing and dividing cells can reduce the quality and quantity of the data collected. Having worked with agar pads for many years before transitioning to microfluidics, we have found it difficult to avoid spatial and temporal heterogeneity in growth conditions – even large effects were often difficult to identify without careful and creative controls. After cells are seeded on agar, several generations are required to adjust to the new conditions; a few generations later, cells start competing for resources, become exposed to increased pressure, and start forming secondary layers. Because many biological processes respond to such changes in conditions, this can cause artefactual variations in molecule abundances that obscure the natural stochasticity in individual cells. Therefore, in our opinion, the reason to avoid agar pads primarily stems from the inability to straightforwardly interpret time-lapse measurements.

However, there is no guarantee that microfluidic devices solve the aforementioned problems, unless thorough steps are taken to ensure spatial and temporal uniformity of the conditions – there is little point in reducing statistical errors if systematic artifacts instead start to dominate. In fact, because of the increased sampling often enabled by microfluidics, the artifacts also become more statistically significant, making it more tempting to interpret them as reflective of underlying biology. Not all microfluidic tools are created equal in this regard, and here we review advantages and disadvantages of recently developed devices, with focus on the last few years and on devices designed for imaging single bacteria rather than sorting or manipulating them. For reviews on related topics, see references [8–14].

Linear colonies

To our knowledge the first study using microfluidics to track the growth of individual bacteria relied on linear grooves in polydimethylsiloxane (PDMS) – a transparent, gas-permeable polymer – which were seeded with a small number of cells and then enclosed by a membrane (Figure 2a) [15]. The cells were fed media diffusively through the membrane while imaged on a microscope, allowing observation of many doublings before the trenches filled up. Compared to agar pads, this technology enabled more uniform feeding as well as the ability to switch media almost instantaneously. The growth of cells in linear colonies also made it easy to keep track of cells throughout the experiment. The device was first used to analyze *Escherichia coli* persisters – a phenomenon where cells stochastically switch between their regular growth state and a rare slow-growing ‘persister’ state that is insensitive to antibiotic treatment. Specifically, an antibiotic was introduced to kill most cells, but when the antibiotic was removed, the progeny of the persister cells eventually became sensitive to the antibiotic again [15]. Since then, persistence has also been investigated in other bacteria, such as *Mycobacterium smegmatis* [16] and *Salmonella typhimurium* [17], using microfluidic devices.

Monolayer colonies

The linear-colony device above was well designed to study persistence, where most cells were killed by antibiotics and growth was monitored for several hours, but one limitation for other applications is that the trenches eventually fill up with cells, limiting the length of the

time series. The first device, to our knowledge, that supported long-term time-lapse fluorescent microscopy of single cells was the monolayer growth chamber chip [18,19]. The design confines growth to a two-dimensional plane, rather than one-dimensional trenches, surrounded by flowing media that ensures both continuous feeding and washing away of peripheral cells (Figure 2b). Early versions of this platform made it possible to characterize multi-generational oscillations in gene expression [20], where very long time series were necessary to assess stability and noise. Similar set-ups, with increased throughput and rapid media-switching capabilities, facilitated studies of how single LacI dimers bound to and dissociated from their cognate operator sites [21]. The fact that cells are in direct proximity to each other is also useful when observing community behaviors [22–26] that require communication through contact or secreted molecules. For example, in a study by Din et al. [23], the microfluidic platform was used to monitor *Salmonella* expressing a bacteriophage gene controlled by a quorum-sensing module, which supported synchronous lysis at a threshold cell density.

However, the monolayer device does suffer from several drawbacks for some applications. One potential pitfall is the lack of uniform conditions across the chamber. While recent studies have reported the absence of spatial patterns in growth rates [19,27], many groups also observe more overall variability in the two-dimensional chambers than what could be achieved for the same cells and media with other devices (see e.g. reference [21]). That has also been our experience when analyzing various genetic circuits: the phenotypic heterogeneity reported using the monolayer device was always much greater than we saw with devices that were tailored to ensure uniform growth. One possible explanation is that microenvironments are created by fluctuating cell movements during proliferation [29]. Either way, in addition to tests that averages and variances are stable over time without spatial biases, we recommend two more tests: comparing snapshots of cells from liquid culture to ensure that the population properties are similar, and testing that properties do not locally correlate more than expected given the cells' genetic distance from a common ancestor. The latter test is complicated by the fact that cells close to each other in space are more likely to have a recent common ancestor, but this can be accounted for by confirming that cells are no more correlated with distantly related cells that happen to live nearby than those located farther away.

Another problem arises even with micro-colonies where every bacterium is equally well fed: fluorescent signals will inevitably be misallocated simply because the length scale of the point-spread function of light (PSF) is similar to the length of the bacteria. Only some of the light emitted can thus be reliably tracked back to the correct cells, which also means that cells with more neighbors tend to appear brighter. In our experience, the main issue is that the tail of the PSF decays slowly with distance. It is then not merely the close neighbors that contribute to spillover signal, but often the large and varying number of distant neighbors.

The mother machine and single cell chemostat: continuous-growth linear colonies

Our favorite devices originated from the Jun and Cluzel groups, where cells grow in linear trenches open to media channels on one or both ends (Figures 2c–d). After the bacteria fill up the trenches, subsequent growth causes additional offspring to be pushed out into the growth medium where they are washed away [30–32]. This makes it possible to maintain a linear mini-colony of constant size in the face of continuous growth and division – about 700 consecutive generations have been observed in our longest experiments [32].

These devices are made of agarose or PDMS, and are attached to coverslips for compatibility with high numerical aperture imaging. The trenches are typically made slightly taller than the cell diameter, to ensure that cells stay close to the coverslip without being squished, and with a width slightly greater than the height, to ensure free space for diffusing media without fitting two cells side by side. The distance between adjacent trenches should be determined by a trade-off between throughput and the PSF issues discussed above. Trenches can be placed very close for greater statistics or separated enough to greatly reduce the spill-over of fluorescence from one cell to the other [32,33]. The length of the channels is in turn often set by a trade-off between growth uniformity and cell retention: the shorter trenches allow for more uniform feeding, but cells then wash out more easily. One way around this tradeoff is to outfit longer trenches with feeding gutters on either side [32], which are made too thin for cells to enter while still allowing nutrients to rapidly diffuse alongside cells. This design was introduced to measure how *Bacillus subtilis* cells stochastically switch between two cell fates – a motile state and a sessile state with multicellular chains (a precursor to biofilm formation). These experiments thus required very uniform growth and long channels for imaging over hundreds of generations. Additionally, the Elf lab has recently engineered devices with small back-end openings, such that media can traverse the trenches while still retaining cells [34], much like versions previously developed for fission yeast [35]. This made it possible to rapidly load bacteria from clinical samples and assess their antibiotic sensitivity.

When these types of devices are closed to cells on one end, which is most common, they are often referred to as ‘mother machines’, based on a pun one of us (J.P.) once made with its inventor, Suckjoon Jun, that the design was conceptually similar to the ‘baby machine’ by Helmstetter and Cooper [36], but now used to study the mother cells that are retained rather than the babies that are washed out. Motherhood is here operationally defined by the age of the pole: the cell closest to the end of the trench is permanently retained and thus gradually grows ‘older’.

The mother machine is indeed an excellent device for studying this type of aging. It keeps track of divisions and can collect data for cells that otherwise would be too old to find in the population. For example, rapidly growing bacteria can go through 100 doublings in a little over a day in the mother machine, producing data for cells of all ages, whereas in an unrestrained population in a flask, only one cell in 10^{30} would then have a pole that is 100 generations old. Observing *E. coli* cells over two hundred generations, Wang et al. showed that the growth rate did not change over time, though the spontaneous filamentation rate and

SOS-dependent death rate increased [30]. More recently, it has been shown that the multi-drug efflux pump AcrAB-TolC exhibits biased partitioning to the old pole in *E. coli* [37]. Mother cells that inherited more of the efflux pump grew faster than cells with younger poles in the presence of subinhibitory antibiotic concentrations. We have observed similar aging phenotypes for some properties, but in our experience, the expression dynamics of most genes – across a wide range of conditions – remain independent of cell age. However, this needs to be checked in every experiment to rule out age-related explanations. When quantitatively comparing results to batch culture, it is also important to account for the fact that the relative frequency of cells at different stages in the cell cycle is different between the mother machine and liquid culture. Specifically, in batch culture, newborn cells are twice as frequent as dividing cells because each dividing cell creates two newborns; this age structure does not hold for mother machine populations.

It is also possible to keep both ends of the growth trenches open to cell passage [31], to avoid aging altogether (Figure 2d). The first such device was made of agarose, which is softer and more generally permeable than PDMS. Agarose also ‘hugs’ the cells more than PDMS, which prevents cell ‘jittering’ – small, fast movements in the liquid suspension – as often required for single-molecule imaging. However, PDMS devices tend to allow for much longer time series. The devices thus need to be tailored to the application.

Using microfluidics to count single molecules

Another microfluidic device that we have found useful for studying stochastic processes in bacteria was developed by Okumus et al. [38]. The cells are grown in conventional liquid culture, pumped into an observation chamber where pressure is applied in a highly controlled manner to collapse the chamber ceiling, and imaged as they are pressed against the coverslip (Figure 2e–f). The full cycle only takes a few seconds and thus fresh batches of cells can be continuously imaged, as many as 10^5 – 10^6 cells per hour. The device, termed ‘MACS’ for microfluidics-assisted cell screening, can operate with a range of different pressures: mild pressures merely bring cells in contact with the coverslip, while higher pressures allow cells to take up drugs that cannot otherwise permeate the cell membrane. The device can also be used to enrich for live cells of interest, akin to fluorescence-activated cell sorting (FACS). However, the most unexpected, and maybe the most useful, feature is that trapping and slightly pressing on cells in a controlled manner appears to expel water from cells and thereby slows down the diffusion of proteins by two orders of magnitude. This means that fluorescent proteins act as if they were mechanically fixed during common camera exposure time-scales, making the fluorescent signal stand out over the local background. The cells are also slightly compressed and widened, which makes it easier to keep all spots in focus, reduces the projected autofluorescence in the z-dimension, and reduces the risk of overlap between fluorescent foci. By using fluorescent protein tandem fusions, counting photobleaching steps, and comparing counts to other methods, it was shown that this method can be used to accurately count individual fluorescent proteins in *E. coli*. The disadvantage of this device is that it does not provide multigenerational time-lapse movies of individual cells.

The first application of MACS focused on the stochasticity of DNA repair in *E. coli* [2], specifically the *ada* pathway where the Ada protein not only repairs DNA methylation damage, but also senses damage to upregulate its own production. Surprisingly, about a third of the cells failed to upregulate Ada levels regardless of the amount of the DNA damaging agent. Single-molecule fluorescence microscopy with MACS in turn revealed that cells on average produced a single Ada molecule per generation before the upregulation and – due to the inherently stochastic nature of gene expression – that about a third of the cells contained zero Ada proteins. Because Ada upregulation requires Ada, the cells were trapped in a state where they could not respond until they randomly produced the first molecule, which took one cell generation on average. Those cells were thus unable to activate the adaptive response that would allow them to repair the damage. In contrast to the classical perspective on why cells fail to repair damage, it was thus not so much the intrinsic randomness of the DNA damage search process that caused failures, but rather the randomness of gene expression. As a result, non-genetic heterogeneity in protein levels is suspected to drive genetic heterogeneity in the population. Other applications of MACS have focused on the stress response. Okumus et al. [38] quantified fluctuations of SprE – the dedicated adapter protein of the master regulator of stress response in *E. coli*, RpoS – and e.g. showed that despite its importance in early exponential phase, it was only present in 3–4 molecules per cell with Poisson statistics.

Cell-size homeostasis

How cells maintain a constant size despite unavoidable fluctuations in the processes of growth and division has been a subject of interest for almost a century (for a recent review, see reference [7]). Microfluidic devices have recently made it possible to accurately measure cell size as they grow and divide under constant conditions [28,39–44]. This in turn made it possible to test different models of cell size control, e.g. whether cells divide when they reach a certain size (“sizer”), after they have grown for certain period of time (“timer”), or after they have added a certain volume (“adder”). These studies have shown that diverse bacterial species, at both intermediate and fast growth rates, add a constant volume per cell division regardless of their size at birth (*E. coli* [28,40,45], *B. subtilis* [45], and *Pseudomonas aeruginosa* [46]). Therefore, deviations in cell size are mostly passively corrected (every cell division, the previous deviation is on average halved by regression to the mean, though new fluctuations also arise), which appears to be in contrast to some higher organisms [7].

By measuring DNA replication in individual cells during growth and division, Wallden et al. [28] came up with a model coupling chromosomal replication and cell division to achieve cell-size homeostasis (a single-cell Cooper-Helmstetter model). They observed that DNA replication initiates at a fixed unit volume per chromosome and predicted that *E. coli* would behave as an adder at fast and intermediate growth rates, but between an adder and a sizer at slow growth, as also observed by others for several bacteria [27,28,39,41,45]. This is a rapidly advancing area of research, and the molecular players underlying homeostatic size control will hopefully soon be identified.

Applications in Synthetic Biology

Synthetic biology has also greatly benefited from microfluidic devices, e.g. to speed up testing, increase sampling, and separate between heterogeneity that arises from random reactions inside cells rather than varying growth environments. For example, many studies have focused on genetic oscillators, starting with the repressilator that helped launch synthetic biology as a field in 2000 [20,22,33,47–52]. The repressilator, the first synthetic oscillator, used a simple design where three genes inhibit the production of each other in a single feedback loop to create oscillations in the levels of each protein. Although this design was previously thought to oscillate more irregularly than other designs, we recently showed that much of the noisy behavior originally reported came from the varying conditions on agar pads, rather than the circuit itself, and that much of the remaining noise came from plasmid vectors and the GFP reporter interfering with the control circuit [33]. By making minor changes, guided by stochastic theory, we also showed that even this simple circuit can create correlated oscillations that persist for hundreds of generations before on average drifting out of phase by half of a period (Figure 2g–h). Microfluidic cell-free systems can also be useful to rapidly test synthetic circuits and to evaluate dynamics in the virtual absence of low copy fluctuations [52].

Conclusion and Future Directions

Microfluidic devices have already enabled many insights across diverse aspects of microbiology, such as antibiotic tolerance and persistence; basic cellular physiology; response to stress such as DNA damage; and synthetic biology. Because these devices permit new ways of looking at cells – allowing us to observe more single cells over longer time, sometimes with single molecule resolution, while maintaining precise control of growth conditions – we expect that they will continue to generate a plethora of knowledge in microbiology as they become adopted by more laboratories.

Future technical developments could further increase throughput to study the many events in cells that are extremely rare, but of great importance, and the physical isolation of single cells from these devices could permit screens of mutant libraries based on dynamic phenotypes. Recent microfluidic devices have also been used to study biofilms [54–57], as well as interactions between bacteria and mammalian cells [23], or bacteria and plant roots [58]. The ability to combine these approaches, to study complex communities such as multi-species ecosystems with changing densities, while maintaining precise control over the environment of the many cells under long-term imaging, will likely lead to a range of new discoveries.

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Highlights

- Advances in microfluidics show that stochastic processes are both ubiquitous and important in bacteria
- Microfluidic culturing devices permit long-term microscopy under tightly controlled conditions
- Microfluidics-assisted cell screening (MACS) enables high-throughput single-cell microscopy with single-molecule sensitivity

Figure 1.

The importance of single-cell and time-lapse measurements. a) Two dose-response mechanisms with identical average behaviors (blue lines), but different single-cell responses (dots, colored by density). On the left, cells progressively respond to the dose following the population average; on the right, cells are either completely on or off, and the fraction of the active population increases with the dose. b) Two stochastic processes can have the same probability distribution, but fluctuate on different timescales. The numbers of molecules are shown fluctuating over time (main panel), where the blue trace fluctuates more quickly than the red trace. Although they have identical probability distributions (inset, top), the two stochastic processes can be distinguished by their autocorrelation functions (inset, bottom).

Figure 2.

Microfluidic devices for single-cell analyses. a) Diagram of the first device to support growth of bacteria in linear colonies with continuous time-lapse imaging. Cells grow and divide in strait grooves etched in PDMS (top panel, top-down view). Trenches are enclosed by a permeable membrane and fed diffusely from flowing media above (bottom panel, cross-sectional view). b) Top-down depiction of monolayer growth device. Bacteria are confined to a two-dimensional monolayer, fed by flowing media on both sides of the colony. c) Top-down schematic of the mother machine device. Cells are confined to dead-end linear trenches and fed by flowing media. Red dotted line represents segmentation of old-pole mother cells; yellow dotted line marks a single lineage with daughter cells sharing the same mother. d) Top-down cartoon of the single-cell chemostat device. Bacteria are geometrically constrained to a single dimension, but no old pole accumulates due to the open-ended structure. Flowing media feeds the trapped bacteria and washes away daughter cells. e) Cross-sectional view of the MACS platform. A soft ceiling is expanded by controlled pressure to trap single bacteria from a liquid culture. Squeezing the cells aids single-molecule detection (inset). f) Single field-of-view from MACS. Bacteria with a constitutive segmentation reporter (red) are captured and imaged with MACS. A rare transient phenotype is observed in yellow. g) Kymograph of mother machine cells with fluorescent protein expression reporting oscillations of a synthetic gene circuit [33]. Images, taken at regular time points, of a single lineage (as marked by the yellow-dotted box in panel c) are concatenated, with time represented on the horizontal axis. h) Single-cell trace from the

mother machine. A reporter of the synthetic oscillator (from panel g) is shown in blue; cell area, capturing growth and division, of the same cell is plotted in red.

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