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REVIEW

Applications of single-cell technology on bacterial analysis

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***Background*: Traditionally, scientists studied microbiology through the manner of batch cultures, to conclude the dynamics or outputs by averaging all individuals. However, as the researches go further, the heterogeneities among the individuals have been proven to be crucial for the population dynamics and fates.**

***Results*: Due to the limit of technology, single-cell analysis methods were not widely used to decipher the inherent connections between individual cells and populations. Since the early decades of this century, the rapid development of microfluidics, fluorescent labelling, next-generation sequencing, and high-resolution microscopy have speeded up the development of single-cell technologies and further facilitated the applications of these technologies on bacterial analysis.**

***Conclusions*: In this review, we summarized the recent processes of single-cell technologies applied in bacterial analysis in terms of intracellular characteristics, cell physiology dynamics, and group behaviors, and discussed how single-cell technologies could be more applicable for future bacterial researches.**

Keywords: single-cell technology; bacterial analysis; fluorescent labelling; next-generation sequencing; microfluidics

Author summary: This review briefs several prevalent single-cell technologies and their recent applications on bacterial quantitative analysis, in terms of intracellular level, single-cell physiology, and group behaviors. And each part is coupled with powerful technologies such as fluorescent labelling, single-cell sequencing, microfluidics, etc. This can provide a quick reference to the researchers who are interested in this field.

INTRODUCTION

Bacteria are abundant everywhere in nature. They play critical roles in our biosphere as cycling elements [1]. Through studying bacteria, many important principals of life have been revealed. For example, the genetic central dogma [2] that explains how the genetic information flows to biosystems, and the Monod equation [3] that provides an important relationship between bacterial growth rate and culturing conditions. With in-depth studies and the development of quantitative techniques, researchers have found that cell-to-cell variabilities prevalently exist, even if the environmental and genetic variances are controlled as much as possible [4]. Actually, recent studies, such as gene expression [5,6], cell growth and division [7], genetic mutation [8] and dCas9 target search [9], have started to focus on sing-cell snapshots gaining more informative details than bulk averages from the batch culture. The results indeed reflected deeper mechanisms underlying the biological processes and reinforced the sing-cell technologies to dissect the heterogeneity among population samples.

In general, single-cell analysis technologies focus on three levels of the cellular heterogeneity: (i) intracellular components, (ii) individual cell dynamics, and (iii) cellular group behaviors. Cellular heterogeneity

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arises from fluctuations in gene expression [10,11], stochastic partition of molecules [12] or the random processes within genetic regulatory networks [13]. To figure out the mystery of heterogeneity, the first step is to quantify the intracellular components regarding their number and localization. Moreover, time autocorrelation behaviors that are associated with cellular heterogeneities are difficult to study within a population due to lacking global synchronization, such as oscillation [14,15] and DNA replication initiation [7,16]. Thus, monitoring individual cells in a high-throughput manner is necessary to depict single-cell dynamics and even population behaviors. In fact, microbial behaviors, like chemotaxis, migration, and communications, have already overstepped our intuition, thus, employing high-quality single-cell data with mathematical models to explore quantitative understanding is becoming more and more important [17]. In this review, we present recent applications of single-cell technologies on bacterial analysis, organized by the aspects of cellular size, in terms of intracellular, single-cell, and group levels (Figure 1).

CHARACTERIZATION OF INTRACELLULAR COMPONENTS

Intracellular components, including DNA, RNA, and protein, etc., are the essential elements that drive the cell to work orderly. These molecules have unique functions, and they interact with each other forming a complex interaction network. For systematically evaluating how these molecules work, we should concern both their properties (*e.g.*, enzymatic activities) and their properties in the network (*e.g.,* quantities, locations, and interaction relationships). Recently, more and more studies suggest that it is incomprehensive to quantify these properties via batch cultures because their variabilities are averaged out in the population level [18]. In this section, two categories of single-cell techniques (Figure 2), which are based on fluorescent labelling and next-generation sequencing, will

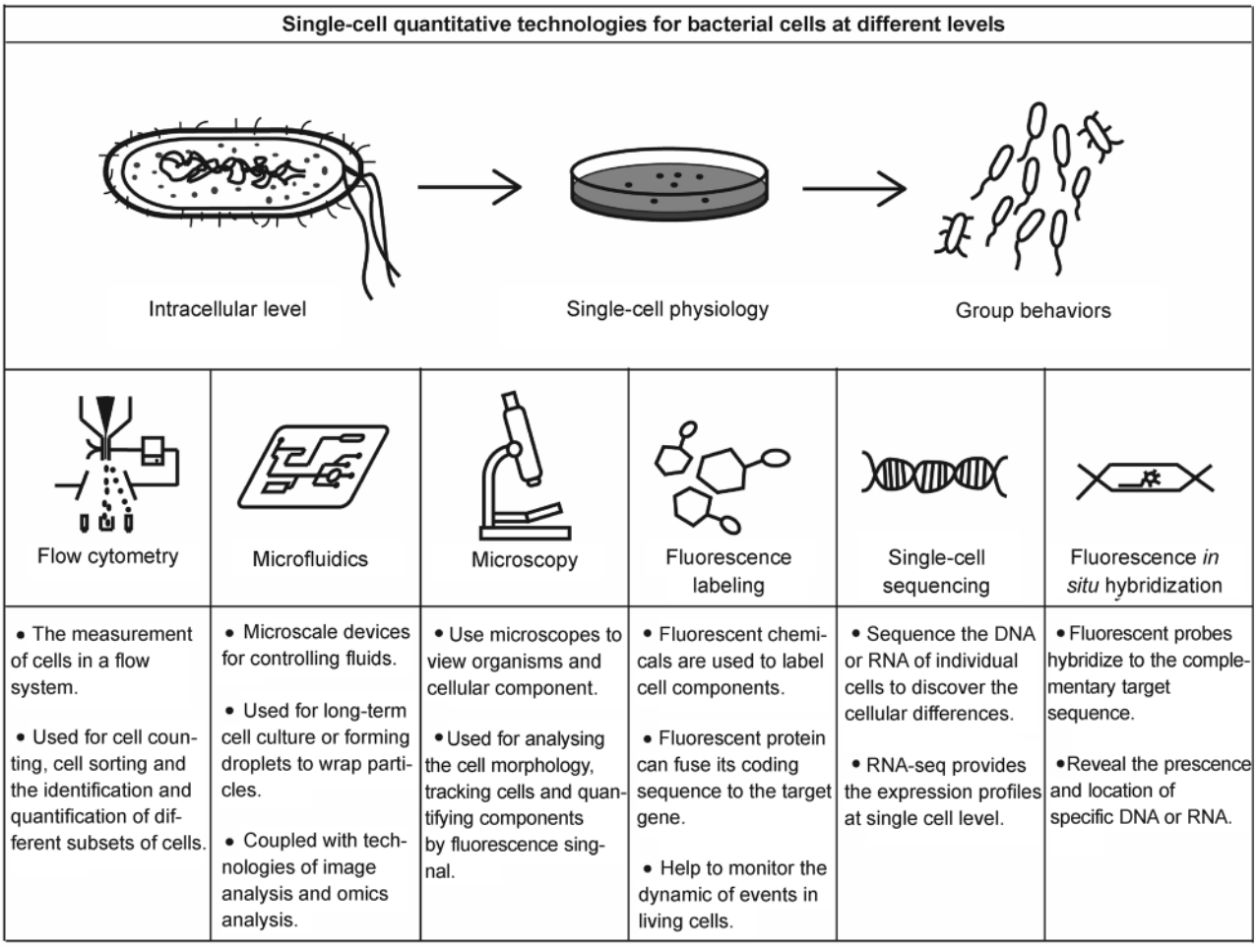

Figure 1. Technologies at different scales for single-cell analysis.

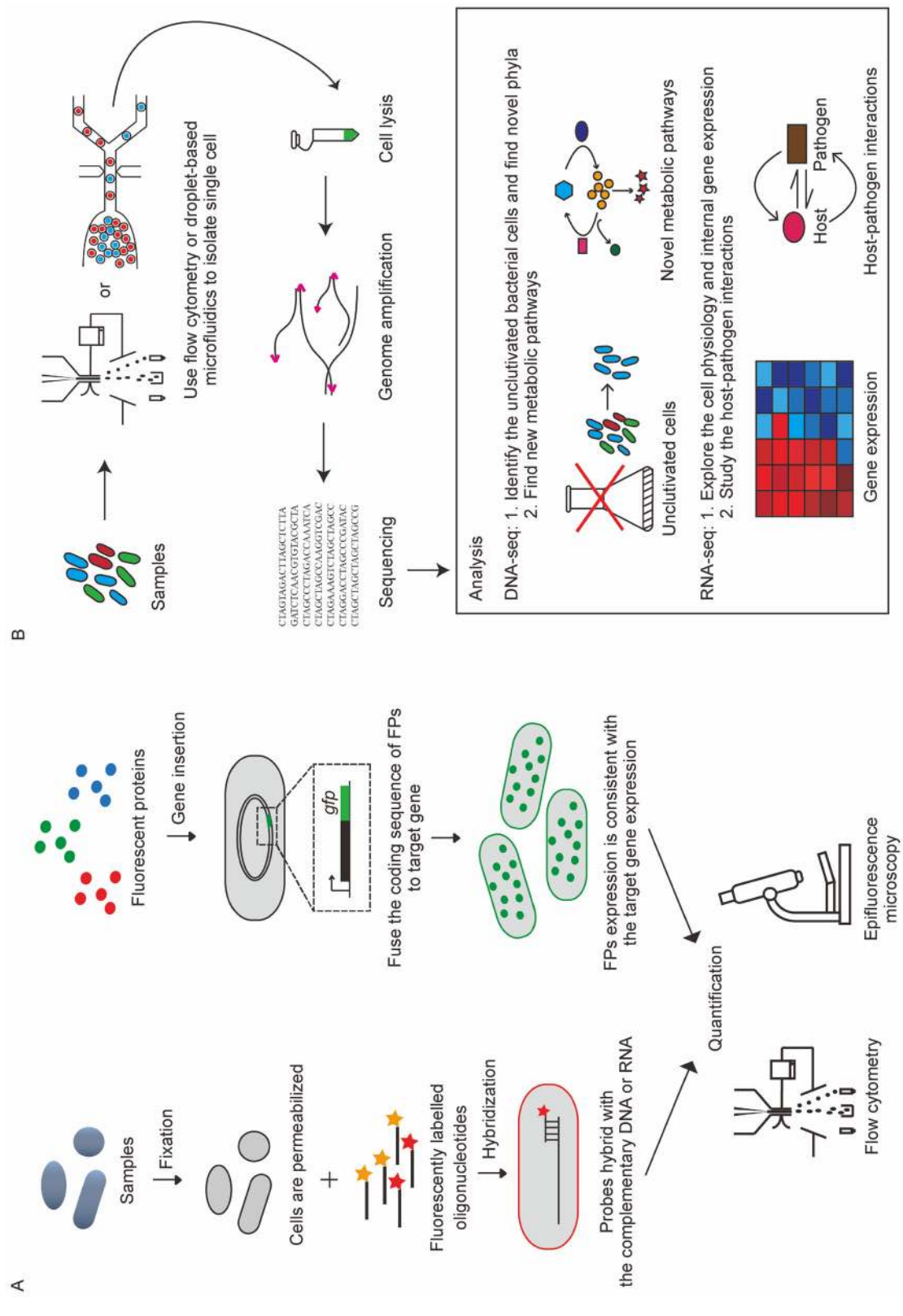

Figure 2. Fluorescent labelling and next-generation sequencing provide visualized and intrinsic information. (A) Traditional processes of fluorescent labeling: aptamers (left) and fluorescent proteins (FPs, right) [19]. (B) Experimental flow for the next-generation sequencing [20]. The typical applications of scDNA-seq and scRNA-seq are included in the box, respectively.

be depicted to show their potential in characterizing intracellular components and uncovering the veil of cellular heterogeneities.

In the bacterial analysis, two kinds of typical fluorescent labelling are frequently quoted and explored, which are oligonucleotide probes and fluorescent proteins (FPs) (Figure 2A). Oligonucleotide probe is normally a short sequence of nucleotides labelled with fluorescent molecules or coupled with reporter molecules, such as fluorescence labels *in situ* hybridization (FISH) and the intrinsic cellular transcripts known as aptamer. In 1989, Delong *et al*. [21] first used FISH in 16S ribosomal RNA to identify single microbial cells and quantified the changes of ribosome content over time. The variants of oligonucleotide probes for different targets of FISH have increased subsequently such as those for rRNA [22], mRNA [23] and genomic DNA [24]. For example, using single-cell mRNA counting FISH, Chong *et al*. [25] found that the intracellular gyrase concentration is responsible for the transcriptional bursting, which is a major source of gene expression noise. Additionally, FISH combined with flow cytometry could also be applied for sorting and quantifying single cells in mixed populations [26]. However, cells would have to be damaged for permeating oligonucleotides when FISH was deployed. Thus, another kind of nondestructive fluorescent labels, aptamers [27], was proposed and employed for labeling specific RNAs in living cells. These labels were identified in the laboratory through the systematic evolution of ligands by exponential enrichment (SELEX). Among them, the first discovered aptamer, named Spinach [27], which resembled the green fluorescent protein (GFP), can be integrated into 5′-end of a transcript and form a genetic loop binding to the cell-permeable fluorescent molecules, and then emit green fluorescence. Nowadays, RNA labeling toolboxes, including Spinach2 [28], Mango [29] and Broccoli [30], have been provided for RNA quantification *in vivo* and applied to analyze single bacterial cells successfully [31].

FPs are a group of proteins that contain chromophores formed *via* the interactions among amino acid residues. The most well-known one, GFP that comes from jellyfish *Aequorea victoria* [32], is now widely used as a marker for genetic characterizations. Owing to non-specific, small size, noninterference, and cofactor-free, GFP enables us to observe the dynamics of events in living cells [33]. Up to date, FPs have been common tools for characterizing cell physiology, genetic parts, gene circuits, gene expression noises, and transcriptional levels, etc. Using FPs to titrate the transcription level by inserting into downstream parts of a specific promoter or integrating into a genetic circuit can be used to quantify properties of genetic objects or annotate cell physiology at the single-cell level. Norman *et al*. [34] utilized two different FPs, GFP and RFP to label different promoters that are regulated *via* a particular genetic pathway to indicate cell states, and revealed that a simple genetic circuit confers cells a tight timing response ability allowing cells to "cooperate". Moreover, FPs based ensemble strategy was also used to dissect temporal regulation of diverse genetic effects at the single-cell level, including SOS response [35], hysteretic response [36], stochastic metabolic state shift [37], gene expression noise [4,38] and bistability of positive feedback circuits [39], etc. These researches improved our understanding of the correlations between cellular phenotypes/genotypes and intrinsic heterogeneities. Fusing FPs with other functional proteins as a tandem form can not only quantify the expression level of the genes of interest, but also indicate mRNA levels and population structures. For example, when cell tumble bias was measured by controlling the expression of YFP fused CheY protein that is a motility associated protein, a steep response curve of CheY protein versus tumble bias was revealed [40]. Similarly, these composite proteins can be applied to indicate DNA damage events [41], track individual replisomes [16], quantify specific RNA abundance [11,42,43] at the single-cell level as well.

Another extremely useful tool for characterizing intracellular components is next-generation sequencing technology (Figure 2B). It enhances our ability to decipher the nucleotide sequences. Traditionally, researchers sequence the complex samples that are obtained from the environment directly and then assemble a large pool of sequencing results to dig out unabundant genomic resources hidden in the microbial world [44]. Because of the serious imbalance in the distribution of bacterial species and the highly complex sequencing database, it is challenging to recognize the genomes of low-abundance microorganisms. For addressing further insights within complex microbial communities, more researchers are seeking physical separation methods, such as flow cytometry [45] and droplet-based microfluidics [46], to isolate single cells from the population for downstream DNA amplification and sequencing. This method, so-called single-cell DNA sequencing (scDNA-seq), could bypass the drawbacks of the traditional sequencing methods and provide new information for biological samples. For example, Rinke *et al*. [46] applied scDNA-seq to target and sequence uncultivated bacterial cells and found novel phyla that were missed by the conventional methods. Moreover, their work also found that a new purine synthesis pathway that was considered belonging to archaea presented in bacterial species, which indicated that there are lateral gene transfers between bacteria and archaea. NGS can also be used to quantify the real-time RNA information *via* counting the sequencing reads of cDNA. This technology, so-called RNA

sequencing (RNA-seq), was widely used to analyze physiological states of live cells. For a decade, sing-cell RNA sequencing (scRNA-seq) was broadly applied to explore the physiology and internal gene expression patterns of eukaryotic cells. However, reports of its applications in prokaryotic cells are scarce up to now. One of the reasons is that the small amount of RNA is normally undetectable, as well as lacking polyadenylated tails on mRNA and short half-life time of RNA [47]. To our knowledge, the first study of prokaryotic single-cell transcriptome analysis was reported by Kang *et al*. [20,48]. In their work, *Burkholderia thailendensis* was used for identifying the gene upregulation or down-regulation in presence of subinhibitory concentrations of glyphosate. Through acquiring the relative transcription level of mRNAs at the single-cell resolution, physiological states of cells were successfully depicted. Thus, this method is quite ingenious in some scenarios, especially for complex and scarce samples, such as bacterial pathogens that have invaded mammalian cells, for example, *Salmonella entrica*. These bacteria will exhibit highly heterogeneities in physiology after infecting host cells. Avital *et al*. [49] mapped the gene regulatory patterns of intercellular pathogen *Salmonella typhimurium* with transcriptomes of the host and found that three different states of pathogens actually behaved in a linear progression. This result generated new insights into the host-pathogen interactions and might open up a new way of antimicrobial treatments [49,50].

SINGLE-CELL BACTERIAL PHYSIOLOGY

Bacterial physiology is a classic and important topic in microbiology [51]. It tries to explain all life processes in an individual bacterium throughout the growth and reproduction. Traditionally, the physiological characteristics come from the batch culture, like grow rates, death rates, and size distributions. Mathematically, the Monod equation even provides a quantitative relation between growth rate and the concentrations of growth-limiting substrates [3]; While nutrient growth law emphasizes correlations between balanced growth and cellular chemical composition in any defined medium [52,53]. Scientists mostly validate these relations by population experiments and employ the average values as the fundamental parameters to represent that of single cells. This can result in homogenization for cellular characteristics, and cover up the heterogeneity among single cells [54]. Every individual bacterial cell differs from another to some extent, but this information is normally ignored partially due to the insufficient understanding. However, when researchers try to dive deeper into the mechanisms underlying the observations, this heterogeneity or noise from single cells can provide powerful supports. And thus, single-cell technologies become more and more attractive for bacterial physiology in recent years.

Time-lapse microscopy is a powerful method for detecting physiological dynamics at the single-cell level [55]. The microscopy platform could be easily coupled with agarose pads for long-term monitoring of single cells (Figure 3A). However, the nutrition quickly depletes due to the fast growth of bacteria, then causing the uneven growth conditions for different partitions on the same pad. To solve this problem, microfluidic chips are comparably ideal, which can provide sustainable nutrition by continuous flow. It can then be used to address an important status of bacterial cells, namely steady state, related to the nutrient growth law [52,53]. Traditionally, the main method to keep this steady state is serial dilution, which is time consuming and labor intensive. In comparison, microfluidics is easier to operate. Briefly, bacteria are loaded into very tiny growing environments on a microfluidic chip, the whole chip is set up with specific culturing conditions and then monitored under a microscope for hours, even a couple of days or weeks depending on the experimental requirements. As reported, there are two main categories of microfluidic designs, which we name “Chamber” and “Mother Machine” (MoMa), besides the other excellent works.

The Chamber design is normally a layout of microfluidic traps that allows bacterial cells growing as a monolayer (Figure 3B). For example, the Elf group designed a typical chamber-like chip inspired by Mather *et al*. [56]. A method combining microfluidics, time-lapsed microscopy, and automated image analysis was developed subsequently [57], which can analyze single-cell intracellular dynamics like gene expressions with a high-throughput manner. Campos *et al*. elaborated homeostasis mechanisms of bacterial cell size using the same design from Elf group [58]. Wallden *et al*. studied the cell size and cell cycle in a further step, and they found that differences in growth rate resulted in cell-to-cell variations for both division timing and cell size; with a similar device, Wehrens *et al*. [59] cultured long filamentous *Escherichia coli* and argued that divisions are controlled by the Min system and the adder principle, respectively. Walkmoto group [60] proposed an empirical growth law that constrains the maximal growth rate of *E. coli* by analyzing the data from a narrower chamber that contains only a few lines of cells.

For the MoMa chips, it is similar to the above chamber design, but with a much smaller feature that allows only one single line of bacterial cells to grow, as shown in Figure 3C. Better than the chamber, MoMa chips can be used to track one single cell, named as the mother cell, for hundreds of generations. By increasing the number of trapping lines, the throughput of mother cells can be substantially promoted as well. This can then provide a

large amount of data point combined with time-lapse monitoring for statistical analysis, and reveal the distribution of single-cell heterogeneities, including cell size, growth rate, death rate, gene expression and so on. For instance, Jun group [7] used the MoMa to investigate robust growth of *E. coli* cells by hundreds of generations for more than 60 hours. It is the first time to make it possible to culture one individual bacterial cell at stable states for a long period. MoMa is regarded as a powerful tool to study bacterial cell-size control as well, due to its high spatiotemporal resolution. Taheri-Araghi *et al*. [61] cultured *E. coli* and *Bacillus subtilis* in MoMa and put forward an adder principle to quantitatively explain the cell-size control and homeostasis mechanism. Sauls *et al*. [62] developed the adder principle and proposed a coarse-grained approach. In contrast, Tanouchi *et al*. [63] proposed a different regulating mechanism, the noisy linear map theory, to explain the origins of cell-size oscillations. MoMa has also been applied to the research of gene expression [55], evolution [64] and cell cycle [34,65]. Besides this, Yang *et al*. [66] systematically characterized the *E. coli* growth dynamics within different sizes of MoMa and provided further opinions about how to choose or design appropriate MoMa chips. Their data have strongly supported MoMa to be applied in the studies of single-cell bacterial physiology. To facilitate the

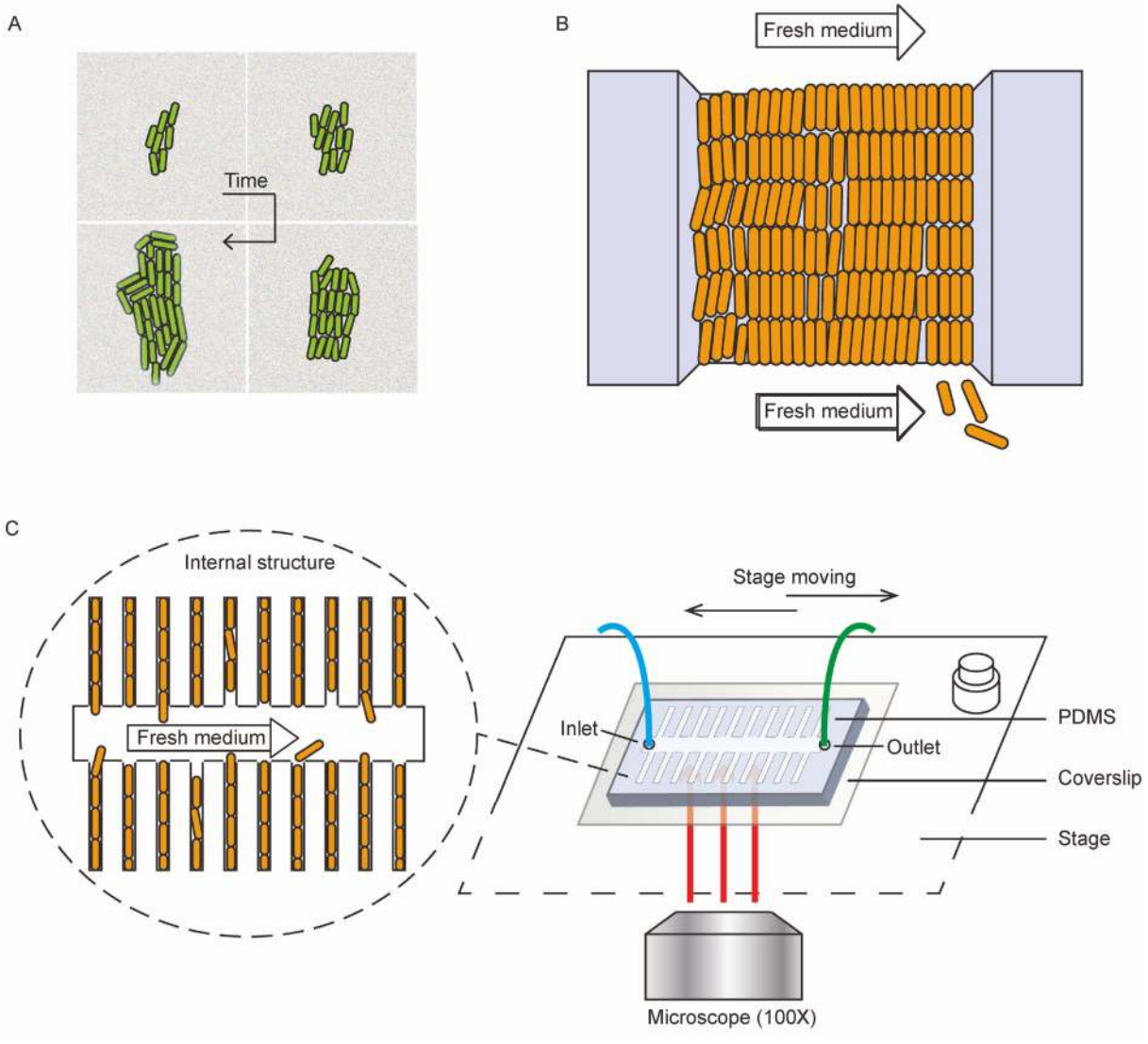

Figure 3. Single-cell observation through microfluidic chips and time-lapse microscopy. (A) Diagram of bacterial time-lapse mosaic. (B) Diagram of chamber design within a microfluidic device. Monolayer cells can be cultured within the chamber and the fresh medium can be sustainably replenished. (C) Diagram of a mother machine (MoMa). Left: mother cells are trapped at the end of the growth channels. Other cells will be pushed to the main channel and flushed out; Right: multiple positions time-lapse imaging can make sure enough data for analysis.

wide application of Moma, Jun group [67] has also concluded a cultivation protocol for MoMa based on their extensive experience, which is helpful to anyone in the field that feels interested in.

GROUP BEHAVIORS

Group behavior is another important aspect of bacterial analysis, which can provide collective information based on single-cell behaviors. Intuitively, the single-cell data are always stochastic and noisy, concluding the populational behavior is quite challenging. However, taking advantage of single-cell technologies, more and more group behaviors have been deciphered by individual cells, and provide fundamentally new theory or answer biological questions from a more statistical angle [68]. For example, despite heterogeneity on chemotaxis, *E. coli* cells could still have spontaneous collective behaviors when migrating, this has been a confusion between single-cell and populational behaviors for a long time. Fu *et al*. [69] resolved this conflict using a microfluidic device consisting of a long channel (Figure 4A), on which cells could perform the traveling bands and be observed precisely (Figure 4B). Except for chemical gradients, thermal gradients may also have strong influences [71,72], and their interplays have been studied preliminarily as well [73].

Another group behavior, bacterial surface adhesion, is also appealing to many researchers. The characterizing methods include microfluidic devices, cell-tracking programs, atomic force microscopies, etc [74], which can all be applied for single-cell analysis. Researchers have ever tried to reduce the amount of bacterium in a group behavior. However, for some studies, it may not be suitable to separate every single cell from each other. For example, Kim *et al*. cultured bacteria within microfluidic wells at a density of 500–1000 live cells/well. They tested a synthetic community comprising three kinds of bacteria. And the results showed that the microscale spatial structure plays an important role in coexistence [75].

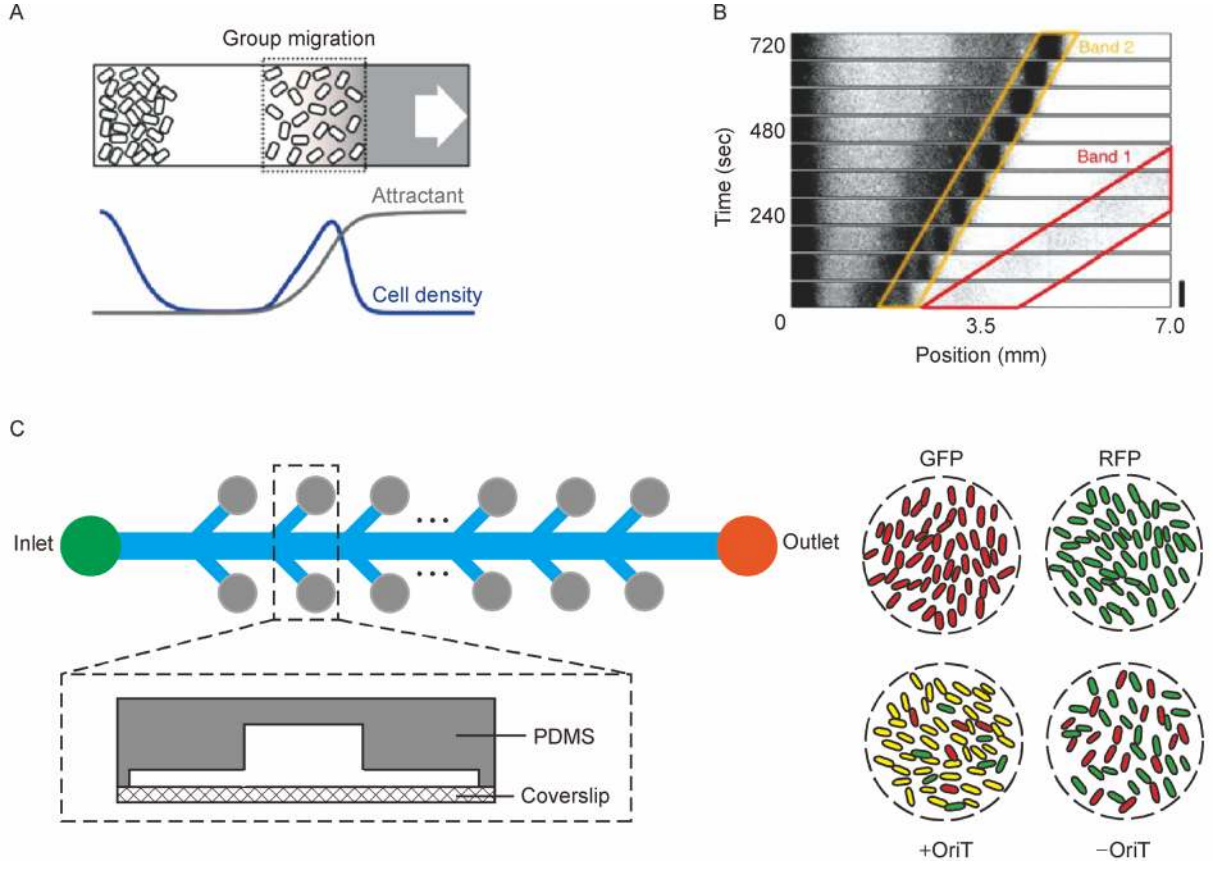

Figure 4. Single-cell technology for bacterial group behaviors. (A) Cells are concentrated at the bottom of a microfluidic channel originally and then travel in bands along the channel following the nutrition attractants (Adapted with permission from Ref. [69]). (B) Cells (black dots) traveling in M9 glycerol medium from (A) to investigate the coordination of single cells for population motility (Adapted with permission from Ref. [69]). (C) Schematics for a novel device to quantify microbial conjugations (Left). The bacterial cells with OriT apparatus could be transferred with another kind of plasmid in the presence of antibiotic treatment, shown as yellow cells (Right). While for the cells without OriT, they only appeared their own color, either red or green [70].

Hence, microscale bacteria population seem to be the least unit for studies on co-culture systems, but the single-cell resolution method provided information about how the community communicated and transported.

For the bacterial group analysis, one inevitable topic is antibiotic resistance [76,77]. The alarming emergence of multidrug resistance (MDR) of bacterial pathogens has been recognized as a global issue. Thus, how to further uncover the mechanism of the generation and prevalence of MDR based on single-cell technologies can help the society control the spread of MDR. One of the most important pathways for resistance dissemination is the bacterial conjugation, which delivers resistant genes from one microorganism to the others. Lopatkin *et al.* [70] developed a novel device that could gain single-cell resolution, and surprisingly, they found that the conjugation efficiency is independent of antibiotic dosing for almost all commercialized types of antibiotics (Figure 4C). Srimani *et al.* [78] utilized the same device to investigate the postantibiotic effect (PAE). PAE refers to the temporary suppression of bacterial growth following transient antibiotic treatment. What they found is that PAE can be explained by the temporal dynamics of drug detoxification in individual cells after an antibiotic is removed from the extracellular environment.

CONCLUSIONS

For a long time, people have to collect bacterial information through macroscale cultivation, commonly based on wells, tubes, flasks, or even larger containers. The results indeed concluded many important biological rules regarding bacterial physiology, gene expression, and regulation etc [3,79–81]. However, deeper mechanisms hidden by a large population have started to be dug out as the rapid development of technologies, and one typical example is the single-cell technologies. For example, the flow cytometry can be applied for the high-throughput detection of single cells [82], but it is not applicable for real-time monitoring. Thus, microscopy can be proposed to supplement the time-scale information. Combining with microfluidics, the growth environments can be well defined, and cultivating medium or stress can also be flexibly controlled [83]. This can mimic more situations in nature and obtain data with much higher resolution than traditional methods. When going deeper into the single cells, the cellular components can also be analyzed to reveal the detailed information for genetic regulations and protein interactions. Herein, we mainly introduced the fluorescent labelling of oligonucleotides and proteins, and next-generation sequencing. These two methods are so far popular assisting tools for single-cell analysis [84].

Although the information has been substantially increased from the current single-cell technologies, there are still a lot of uncovered rules and information needed to be figured out. We believed that more and more novel technologies would be integrated for bacterial analysis, for instance, CRISPR [85] for intracellular genetic locations and super-resolution microscopy [86] for even intracellular structures, to decipher the single cell information [87,88] and then population behaviors and community interactions. Many studies hereinbefore cited have tried to answer bacterial fundamental questions using single-cell technologies, but their potential is sought to be further explored for expanding our understanding of sophisticated life processes of bacteria.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Zhixin Ma, Pan M. Chu, Yingtong Su, Yue Yu, Hui Wen, Xiongfei Fu and Shuqiang Huang declare that they have no conflict of interests.

This article is a review article and does not contain any studies with human or animal subjects performed by any of the authors.

REFERENCES

1. Cryan J. F. and Dinan T. G. (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci., 13, 701–712
2. Crick F. (1970) Central dogma of molecular biology. Nature, 227, 561–563
3. Monod J. (1949) The growth of bacterial cultures. Annu. Rev. Microbiol., 3, 371–394
4. Elowitz M. B., Levine A. J., Siggia E. D. and Swain P. S. (2002) Stochastic gene expression in a single cell. Science, 297, 1183–1186
5. Ozbudak E. M., Thattai M., Kurtser I., Grossman A. D. and van Oudenaarden A. (2002) Regulation of noise in the expression of a single gene. Nat. Genet., 31, 69–73
6. Rosenfeld N., Young J. W., Alon U., Swain P. S. and Elowitz M. B. (2005) Gene regulation at the single-cell level. Science, 307, 1962–1965
7. Wang P., Robert L., Pelletier J., Dang W. L., Taddei F., Wright A. and Jun S. (2010) Robust growth of *Escherichia coli.* Curr. Biol., 20, 1099–1103
8. Robert L., Ollion J., Robert J., Song X., Matic I. and Elez M. (2018) Mutation dynamics and fitness effects followed in single cells. Science, 359, 1283–1286
9. Jones D. L., Leroy P., Unoson C., Fange D., Ćurić V., Lawson M. J.

and Elf J. (2017) Kinetics of dCas9 target search in *Escherichia coli.* Science, 357, 1420–1424
10. Jones D. L., Brewster R. C. and Phillips R. (2014) Promoter architecture dictates cell-to-cell variability in gene expression. Science, 346, 1533–1536
11. Golding I., Paulsson J., Zawilski S. M. and Cox E. C. (2005) Real-time kinetics of gene activity in individual bacteria. Cell, 123, 1025–1036
12. Huh D. and Paulsson J. (2011) Non-genetic heterogeneity from stochastic partitioning at cell division. Nat. Genet., 43, 95–100
13. Chen Y., Kim J. K., Hirning A. J., Josić K. and Bennett M. R. (2015) Emergent genetic oscillations in a synthetic microbial consortium. Science, 349, 986–989
14. Ishiura M., Kutsuna S., Aoki S., Iwasaki H., Andersson C. R., Tanabe A., Golden S. S., Johnson C. H. and Kondo T. (1998) Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. Science, 281, 1519–1523
15. Elowitz M. B. and Leibler S. (2000) A synthetic oscillatory network of transcriptional regulators. Nature, 403, 335–338
16. Wallden M., Fange D., Lundius E. G., Baltekin Ö. and Elf J. (2016) The synchronization of replication and division cycles in individual *E. coli* cells. Cell, 166, 729–739
17. Amir A. and Balaban N. Q. (2018) Learning from noise: how observing stochasticity may aid microbiology. Trends Microbiol., 26, 376–385
18. Prakadan S. M., Shalek A. K. and Weitz D. A. (2017) Scaling by shrinking: empowering single-cell "omics" with microfluidic devices. Nat. Rev. Genet., 18, 345–361
19. Amann R. and Fuchs B. M. (2008) Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol., 6, 339–348
20. Kang Y., McMillan I., Norris M. H. and Hoang T. T. (2015) Single prokaryotic cell isolation and total transcript amplification protocol for transcriptomic analysis. Nat. Protoc., 10, 974–984
21. DeLong E. F., Wickham G. S. and Pace N. R. (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science, 243, 1360–1363
22. Manz W., Szewzyk U., Ericsson P., Amann R., Schleifer K. H. and Stenström T. A. (1993) *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol., 59, 2293–2298
23. Wagner M., Schmid M., Juretschko S., Trebesius K. H., Bubert A., Goebel W. and Schleifer K. H. (1998) *In situ* detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes.* FEMS Microbiol. Lett., 160, 159–168
24. Zwirglmaier K., Ludwig W. and Schleifer K. H. (2004) Recognition of individual genes in a single bacterial cell by fluorescence *in situ* hybridization–RING-FISH. Mol. Microbiol., 51, 89–96
25. Chong S., Chen C., Ge H. and Xie X. S. (2014) Mechanism of transcriptional bursting in bacteria. Cell, 158, 314–326
26. Wallner G., Amann R. and Beisker W. (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry, 14, 136–143
27. Paige J. S., Wu K. Y. and Jaffrey S. R. (2011) RNA mimics of green fluorescent protein. Science, 333, 642–646
28. Strack, R. L., Disney, M. D. and Jaffrey, S. R. (2013) A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat-containing RNA. Nat. Methods, 10, 1219–1224
29. Dolgosheina, E. V., Jeng, S. C., Panchapakesan, S. S. S., Cojocaru R., Chen P. S., Wilson P. D., Hawkins N., Wiggins P. A. and Unrau P. J. (2014) RNA mango aptamer-fluorophore: a bright, high-affinity complex for RNA labeling and tracking. ACS Chem. Biol., 9, 2412–2420
30. Filonov, G. S., Moon, J. D., Svensen, N. and Jaffrey, S. R. (2014) Broccoli: rapid selection of an RNA mimic of green fluorescent protein by fluorescence-based selection and directed evolution. J. Am. Chem. Soc., 136, 16299–16308
31. Arora A., Sunbul M. and Jäschke A. (2015) Dual-colour imaging of RNAs using quencher- and fluorophore-binding aptamers. Nucleic Acids Res., 43, e144
32. Shimomura, O., Johnson, F. H. and Saiga, Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J. Cell. Comp. Physiol., 59, 223–239
33. Stearns T. (1995) Green fluorescent protein. The green revolution. Curr. Biol., 5, 262–264
34. Norman T. M., Lord N. D., Paulsson J. and Losick R. (2013) Memory and modularity in cell-fate decision making. Nature, 503, 481–486
35. Friedman N., Vardi S., Ronen M., Alon U. and Stavans J. (2005) Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. PLoS Biol., 3, e238
36. Ozbudak E. M., Thattai M., Lim H. N., Shraiman B. I. and Van Oudenaarden A. (2004) Multistability in the lactose utilization network of *Escherichia coli.* Nature, 427, 737–740
37. Rosenthal A. Z., Qi Y., Hormoz S., Park J., Li S. H.-J. and Elowitz M. B. (2018) Metabolic interactions between dynamic bacterial subpopulations. eLife, 7, 1–18
38. Taniguchi Y., Choi P. J., Li G. W., Chen H., Babu M., Hearn J., Emili A. and Xie X. S. (2010) Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. Science, 329, 533–538
39. Tan C., Marguet P. and You L. (2009) Emergent bistability by a growth-modulating positive feedback circuit. Nat. Chem. Biol., 5, 842–848
40. Cluzel P., Surette M. and Leibler S. (2000) An ultrasensitive bacterial motor revealed by monitoring signaling proteins in single cells. Science, 287, 1652–1655
41. Uphoff S., Lord N. D., Okumus B., Potvin-Trottier L., Sherratt D. J. and Paulsson J. (2016) Stochastic activation of a DNA damage response causes cell-to-cell mutation rate variation. Science, 351, 1094–1097
42. Badrinarayanan A., Reyes-Lamothe R., Uphoff S., Leake M. C. and Sherratt D. J. (2012) *In vivo* architecture and action of bacterial structural maintenance of chromosome proteins. Science, 338, 528–531

43. Le T. T., Harlepp S., Guet C. C., Dittmar K., Emonet T., Pan T. and Cluzel P. (2005) Real-time RNA profiling within a single bacterium. Proc. Natl. Acad. Sci. USA, 102, 9160–9164
44. Gawad C., Koh W. and Quake S. R. (2016) Single-cell genome sequencing: current state of the science. Nat. Rev. Genet., 17, 175–188
45. Podar M., Abulencia C. B., Walcher M., Hutchison D., Zengler K., Garcia J. A., Holland T., Cotton D., Hauser L. and Keller M. (2007) Targeted access to the genomes of low-abundance organisms in complex microbial communities. Appl. Environ. Microbiol., 73, 3205–3214
46. Rinke C., Schwientek P., Sczyrba A., Ivanova N. N., Anderson I. J., Cheng J. F., Darling A., Malfatti S., Swan B. K., Gies E. A., *et al.* (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature, 499, 431–437
47. Zhang Y., Gao J., Huang Y. and Wang J. (2018) Recent developments in single-Cell RNA-seq of microorganisms. Biophys. J., 115, 173–180
48. Kang Y., Norris M. H., Zarzycki-Siek J., Nierman W. C., Donachie S. P. and Hoang T. T. (2011) Transcript amplification from single bacterium for transcriptome analysis. Genome Res., 21, 925–935
49. Avital G., Avraham R., Fan A., Hashimshony T., Hung D. T. and Yanai I. (2017) scDual-seq: mapping the gene regulatory program of *Salmonella* infection by host and pathogen single-cell RNA-sequencing. Genome Biol., 18, 200
50. Saliba A. E., Li L., Westermann A. J., Appenzeller S., Stapels D. A., Schulte L. N., Helaine S. and Vogel J. (2016) Single-cell RNA-seq ties macrophage polarization to growth rate of intracellular *Salmonella.* Nat. Microbiol., 2, 16206
51. Gale E. F. (2009) Bacterial Physiology. Vol. 2, 1st edition. Elsevier
52. Kjeldgaard N. O., Maaloe O. and Schaechter M. (1958) The transition between different physiological states during balanced growth of *Salmonella typhimurium.* J. Gen. Microbiol., 19, 607–616
53. Schaechter M., Maaloe O. and Kjeldgaard N. O. (1958) Dependency on medium and temperature of cell size and chemical composition during balanced grown of *Salmonella typhimurium.* J. Gen. Microbiol., 19, 592–606
54. Brehm-Stecher B. F. and Johnson E. A. (2004) Single-cell microbiology: tools, technologies, and applications. Microbiol. Mol. Biol. Rev., 68, 538–559
55. Young J. W., Locke J. C., Altinok A., Rosenfeld N., Bacarian T., Swain P. S., Mjolsness E. and Elowitz M. B. (2011) Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. Nat. Protoc., 7, 80–88
56. Mather W., Mondragón-Palomino O., Danino T., Hasty J. and Tsimring L. S. (2010) Streaming instability in growing cell populations. Phys. Rev. Lett., 104, 208101
57. Ullman G., Wallden M., Marklund E. G., Mahmutovic A., Razinkov I. and Elf J. (2013) High-throughput gene expression analysis at the level of single proteins using a microfluidic turbidostat and automated cell tracking. Philos. Trans. R Soc. B Biol. Sci., 368
58. Campos M., Surovtsev I. V., Kato S., Paintdakhi A., Beltran B., Ebmeier S. E. and Jacobs-Wagner C. (2014) A constant size extension drives bacterial cell size homeostasis. Cell, 159, 1433–1446
59. Wehrens M., Ershov D., Rozendaal R., Walker N., Schultz D., Kishony R., Levin P. A. and Tans S. J. (2018) Size laws and division ring dynamics in filamentous *Escherichia coli* cells. Curr. Biol., 28, 972–979.e5
60. Hashimoto M., Nozoe T., Nakaoka H., Okura R., Akiyoshi S., Kaneko K., Kussell E. and Wakamoto Y. (2016) Noise-driven growth rate gain in clonal cellular populations. Proc. Natl. Acad. Sci. USA, 113, 3251–3256
61. Taheri-Araghi S., Bradde S., Sauls J. T., Hill N. S., Levin P. A., Paulsson J., Vergassola M. and Jun S. (2015) Cell-size control and homeostasis in bacteria. Curr. Biol., 25, 385–391
62. Sauls J. T., Li D. and Jun S. (2016) Adder and a coarse-grained approach to cell size homeostasis in bacteria. Curr. Opin. Cell Biol., 38, 38–44
63. Murata A., Isoda K., Ikeuchi T., Matsui T., Shiraishi F. and Oba M. (2016) Classification method of severe accident condition for the development of severe accident instrumentation and monitoring system in nuclear power plant. J. Nucl. Sci. Technol., 53, 870–877
64. Van Houten B. and Kad N. M. (2018) Single-cell mutagenic responses and cell death revealed in real time. Proc. Natl. Acad. Sci. USA, 115, 7168–7170
65. Osella M., Nugent E. and Cosentino Lagomarsino M. (2014) Concerted control of *Escherichia coli* cell division. Proc. Natl. Acad. Sci. USA, 111, 3431–3435
66. Yang D., Jennings A. D., Borrego E., Retterer S. T. and Männik J. (2018) Analysis of factors limiting bacterial growth in PDMS mother machine devices. Front. Microbiol., 9, 871
67. Taheri-Araghi S. and Jun S. (2015) Single-cell cultivation in microfluidic devices. Can. Vet. J., 11, 5–16
68. Martins B. M. C. and Locke J. C. W. (2015) Microbial individuality: how single-cell heterogeneity enables population level strategies. Curr. Opin. Microbiol., 24, 104–112
69. Fu X., Kato S., Long J., Mattingly H. H., He C., Vural D. C., Zucker S. W. and Emonet T. (2018) Spatial self-organization resolves conflicts between individuality and collective migration. Nat. Commun., 9, 2177
70. Lopatkin A. J., Huang S., Smith R. P., Srimani J. K., Sysoeva T. A., Bewick S., Karig D. K. and You L. (2016) Antibiotics as a selective driver for conjugation dynamics. Nat. Microbiol., 1, 16044
71. Yoney A. and Salman H. (2015) Precision and variability in bacterial temperature sensing. Biophys. J., 108, 2427–2436
72. Murugesan N., Panda T. and Das S. K. (2016) Effect of gold nanoparticles on thermal gradient generation and thermotaxis of *E. coli* cells in microfluidic device. Biomed. Microdevices, 18, 53
73. Murugesan N., Dhar P., Panda T. and Das S. K. (2017) Interplay of chemical and thermal gradient on bacterial migration in a diffusive microfluidic device. Biomicrofluidics, 11, 024108
74. Berne C., Ellison C. K., Ducret A. and Brun Y. V. (2018) Bacterial adhesion at the single-cell level. Nat. Rev. Microbiol., 16, 616–627

75. Kim H. J., Boedicker J. Q., Choi J. W. and Ismagilov R. F. (2008) Defined spatial structure stabilizes a synthetic multispecies bacterial community. Proc. Natl. Acad. Sci. USA, 105, 18188–18193
76. Kohanski M. A., Dwyer D. J. and Collins J. J. (2010) How antibiotics kill bacteria: from targets to networks. Nat. Rev. Microbiol., 8, 423–435
77. Meredith H. R., Srimani J. K., Lee A. J., Lopatkin A. J. and You L. (2015) Collective antibiotic tolerance: mechanisms, dynamics and intervention. Nat. Chem. Biol., 11, 182–188
78. Srimani J. K., Huang S., Lopatkin A. J. and You L. (2017) Drug detoxification dynamics explain the postantibiotic effect. Mol. Syst. Biol., 13, 948
79. Zwietering M. H., Jongenburger I., Rombouts F. M. and van 't Riet K. (1990) Modeling of the bacterial growth curve. Appl. Environ. Microbiol., 56, 1875–1881
80. Kargi F. (2009) Re-interpretation of the logistic equation for batch microbial growth in relation to Monod kinetics. Lett. Appl. Microbiol., 48, 398–401
81. Scott M., Gunderson C. W., Mateescu E. M., Zhang Z. and Hwa T. (2010) Interdependence of cell growth and gene expression: origins and consequences. Science, 330, 1099–1102
82. Fulwyler M. J. (1965) Electronic separation of biological cells by volume. Science, 150, 910–911
83. Moffitt J. R., Lee J. B. and Cluzel P. (2012) The single-cell chemostat: an agarose-based, microfluidic device for high-throughput, single-cell studies of bacteria and bacterial communities. Lab Chip, 12, 1487–1494
84. Balleza E., Kim J. M. and Cluzel P. (2018) Systematic characterization of maturation time of fluorescent proteins in living cells. Nat. Methods, 15, 47–51
85. Knott G. J. and Doudna J. A. (2018) CRISPR-Cas guides the future of genetic engineering. Science, 361, 866–869
86. Schermelleh L., Ferrand A., Huser T., Eggeling C., Sauer M., Biehlmaier O. and Drummen G. P. C. (2019) Super-resolution microscopy demystified. Nat. Cell Biol., 21, 72–84
87. Gurjav U., Jelfs P., Hill-Cawthorne G. A., Marais B. J. and Sintchenko V. (2016) Genotype heterogeneity of *Mycobacterium tuberculosis* within geospatial hotspots suggests foci of imported infection in Sydney, Australia. Infect. Genet. Evol., 40, 346–351
88. Ackermann M. (2015) A functional perspective on phenotypic heterogeneity in microorganisms. Nat. Rev. Microbiol., 13, 497–508

