Scale Invariance of Cell Size Fluctuations in Starving Bacteria

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Scale invariance of cell size fluctuations in starving bacteria

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In stable environments, cell size fluctuations are thought to be governed by simple physical principles, as suggested by recent findings of scaling properties. Here, by developing a novel microfluidic device and using *E. coli*, we investigate the response of cell size fluctuations against starvation. By abruptly switching to non-nutritious medium, we find that the cell size distribution changes but satisfies scale invariance: the rescaled distribution is kept unchanged and determined by the growth condition before starvation. These findings are underpinned by a model based on cell growth and cell cycle. Further, we numerically determine the range of validity of the scale invariance over various characteristic times of the starvation process. Combining theory and simulations, we reveal the number of multifork replications is crucial for the scale invariance. Our results emphasize the importance of intrinsic cellular cycle processes in this problem, suggesting different distribution trends for bacteria and eukaryotes.

INTRODUCTION

Recent studies on microbes in the steady growth phase suggested that the cellular body size fluctuations may be governed by simple physical principles. For instance, Giometto *et al.* [1] proposed that size fluctuations of various eukaryotic cells are governed by a common distribution function, if the cell sizes of a given species are normalized by their mean value (see also [2]). In other words, the distribution of cell volumes v , $p(v)$, can be described as follows:

$$p(v) = v^{-1} F(v/V), \quad (1)$$

with a function $F(\cdot)$ and $V = \langle v \rangle$ being the mean cell volume. This property of distribution is often called scale invariance. Interestingly, this finding can account for power laws of community size distributions, i.e., the size distribution of all individuals regardless of species, which were observed in various natural ecosystems [3, 4]. Scale invariance akin to Eq. (1) was also found for bacteria [5, 6] for each cell age, and the function $F(\cdot)$ was shown to be robust against changes in growth conditions such as the temperature.

Those results, as well as theoretical models proposed in this context [1, 7], have been obtained under steady environments, for which our understanding of single-cell growth statistics has also been significantly deepened recently [8–10]. By contrast, it is unclear whether such a simple concept as scale invariance is valid under time-dependent conditions, where different regulations of cell cycle kinetics may come into play in response to environmental variations. In particular, when bacterial cells enter the stationary phase from the exponential growth phase, they undergo reductive division, during which both the typical cell size and the amount of DNA per cell decrease [11–14]. Although this behavior itself is commonly observed in test tube cultivation, little is known

about single-cell statistical properties during the transient. The bacterial reductive division is therefore an important stepping stone for studying cell size statistics under time-dependent environments and understanding the robustness of the scale invariance against environmental changes.

It has been, however, a challenge to observe large populations of bacteria under uniform yet time-dependent growth conditions. For steady conditions, the Mother Machine [15], which allows for tracking of bacteria trapped in short narrow channels, was proved to be a powerful tool for measuring cell size statistics. In such experiments, the channel width needs to be adapted to cell widths in a given condition, and this renders the application to time-dependent conditions difficult. If we enlarge the channels, depletion of nutrients in deeper regions of the channels induces spatial heterogeneity, as discussed in ref.[16, 17] and later in this article. Hence, it is also required to develop a system that can uniformly control non-steady environments and give a sufficient amount of single-cell statistics with high efficiency.

In this study, we establish a novel microfluidic device, which we name the “extensive microperturbation system” (EMPS). This system can trap dense bacterial populations in a wide quasi-two-dimensional region and uniformly control the culture condition for a long time. We confirm that bacteria can freely swim and grow inside, and evaluate the uniformity and the switching efficiency of the culture condition. Then we use this system for quantitative observations of bacterial reductive division processes, triggered by abrupt switching to non-nutritious medium. We observe *Escherichia coli* cells and find that the distribution of cell volumes, collected irrespective of cell ages, maintain the scale invariance as in Eq. (1) at each time, with the mean cell size that gradually decreases. On the other hand, the rescaled distribution function F is found to depend on the growth condition

FIG. 1. Sketch of the extensive microperfusion system (EMPS). **a** Entire view of the device. Microwells are created on a glass coverslip. We attach a PDMS pad on the coverslip with a square frame seal to fill the system with liquid medium. **b** Cross-sectional view inside the PDMS pad. A PET-cellulose bilayer porous membrane is attached via the biotin-streptavidin bonding.

before starvation, slightly but significantly. To obtain theoretical insights on these experimental findings, we devise a cell cycle model describing reductive division processes, by extending the Cooper-Helmstetter model [18–20] for steady growth environments. We numerically find that this model indeed shows the scale invariance under starvation conditions, confirming the robustness of this property. We also provide theoretical descriptions on the time evolution of the cell size distribution, and propose a condition for the scale invariance. Finally, we numerically show the range of validity of the scale invariance over various characteristic times of the starvation process, revealing the number of multifork replications is crucial for the scale invariance.

RESULTS

Development of the extensive microperfusion system

In order to achieve uniformly controlled environments with dense bacterial suspensions, we adopt a perfusion system as developed in ref. [21]. This system allows for supplying fresh medium through a porous membrane attached over the entire observation area, instead of supplying from an open end as polydimethylsiloxane (PDMS)-based systems usually do. In its prototypical setup, bacteria are confined in microwells made on a coverslip, covered by a cellulose porous membrane attached to the coverslip via biotin-streptavidin bonding. The pore size of the membrane is chosen so that it can confine bacteria and also that it can exchange nutrients and waste substances across the membrane. To continuously perfuse the system with fresh medium, a PDMS pad with a bubble trap is attached above the membrane by a two-sided frame seal (Fig. 1a and Supplementary Fig. 1a). This setup can maintain a spatially homogeneous environment for cell populations in each microwell, in particular if the microwells are sufficiently shallow so that all cells remain near the membrane. However, because the soft cellulose

membrane may droop and adhere to the bottom for wide and shallow microwells, the horizontal size of such quasi-two-dimensional microwells has been limited up to a few tens of micrometers, preventing from characterization of the instantaneous cell size distribution.

By the EMPS, we overcome this problem and realize quasi-two-dimensional wells sufficiently large for statistical characterization of cell populations. This is made possible by introducing a bilayer membrane, where the cellulose membrane is sustained by a polyethylene terephthalate (PET) porous membrane via biotin-streptavidin bonding (Fig. 1b, Supplementary Fig. 1b and Methods). Because the PET membrane is more rigid than the cellulose membrane, we can realize extended area without bending of the membrane. Specifically, in our setup with circular wells of 110 μm diameter and 1.1 μm depth, although a cellulose membrane alone is bent and adheres to the bottom of the well (Supplementary Fig. 1c and Supplementary Movie 1), our PET-cellulose bilayer membrane keeps flat enough so that bacteria can freely swim in the shallow well (Supplementary Fig. 1d and Supplementary Movie 2). The EMPS can realize such observations for a long time with little hydrodynamic perturbation by medium flow and no mechanical stress, which may exist in a PDMS-based device that holds cells mechanically. We also check whether the additional PET membrane may affect the growth condition of cells, by using *E. coli* MG1655 and M9 medium with glucose and amino acids (Glc+a.a.). We find that the doubling time of the cell population is 59 ± 10 min, which is comparable to that in the previous system without the PET membrane [21–23]. Therefore, our bilayer membrane can still exchange medium efficiently.

We then test the spatial uniformity of the culture condition. We design U-shape traps with an open end, for both the EMPS (Fig. 2a and for the conventional PDMS-based device (Supplementary Fig. 2a,b). With this geometry, nutrients are supplied via diffusion from the open end in the PDMS-based system, while nutritious medium is directly and uniformly delivered through the membrane above the trap in the EMPS. When we culture *E. coli* MG1655 in M9(Glc+a.a.), the trap is eventually filled with cells, and they exhibit coherent flow toward the open end due to the cell growth and proliferation (Fig. 2b and Supplementary Movie 3,4). To evaluate the uniformity of the cell growth, we measure the velocity field of the cell flow by particle image velocimetry (PIV) (Supplementary Fig. 2c,d). The velocity component along the stream-wise direction (the y axis in Fig. 2b) averaged over the span-wise direction (the x axis), $u(y) = \langle u(x,y) \rangle_x$, clearly shows that the velocity profile is stable for the EMPS over long time periods, while it gradually decreases for the PDMS-based device (Fig. 2c Main Panel and Inset, respectively). The cell growth rate is then obtained by $\lambda(y) = \langle \frac{du(x,y)}{dy} \rangle_x$, which is shown in Fig. 2d. The result shows that the growth

FIG. 2. Cell growth measurements in U-shape traps in EMPS. **a** Sketch of the design of microchannels. Non-motile cells are trapped in the shallow observation area. Cells in the trap can escape to the deep drain channel. **b** Top view of the trap ($30 \mu\text{m} \times 80 \mu\text{m}$, $1.0 \mu\text{m}$ depth) filled with *E. coli* W3110 ΔfliC Δflu ΔfimA (see also Supplementary Movie 4). The scale bar is $25 \mu\text{m}$. Coherent flow of cells driven by cell proliferation is directed toward the drain ($15 \mu\text{m}$ depth). **c** The stream-wise (y) component of the velocity field averaged over the span-wise (x) direction (the two-dimensional velocity field is shown in Supplementary Fig. 2c), $u(y) = \langle u(x, y) \rangle_x$, measured in different time periods. The data were taken from a single trap. $t = 0$ is the time at which the trap is filled with cells. Error bars represent the standard error, which we roughly evaluate by picking up 5 data points along the span-wise direction, at intervals of roughly $5 \mu\text{m}$, half of the size of the PIV window. The open end is located near $y \approx 81 \mu\text{m}$. (Inset) The same quantity measured for a PDMS-based device with a similar trap (Supplementary Fig. 2a,b,c). The open end is at $y \approx 88 \mu\text{m}$. Time dependence is clearly observed. **d** The growth rate profiles λ , evaluated by $\lambda(y) = \langle \frac{du(x, y)}{dy} \rangle_x$, for the EMPS (main panel) and the PDMS-based device (inset). Error bars represent the standard error of the ensemble.

rate λ is indeed uniform and kept constant for the EMPS (Fig. 2d, Main Panel), while for the PDMS device it is heterogeneous, being higher near the open end (Fig. 2d, Inset), and it decreases as time elapses. The growth rate decays at the distance of roughly $30 \mu\text{m}$ from the open end, which is located near $y \approx 88 \mu\text{m}$ for the PDMS device. This observation is consistent with the nutrient depletion length we evaluate by following the calculation by Mather et al. [17], $32 \mu\text{m}$, for which we used the diffusion constant of glucose [24] and the division time of 60 min. Such heterogeneity is not seen in the EMPS. While cell growth regulation pathways may also be influenced by such factors as mechanical pressure caused by cell elongation [25–28], quorum sensing [29, 30], etc., our results indicate that the EMPS can indeed realize a uniform and stable culture condition while the same medium is kept supplied.

Another advantage of the EMPS is that we can also switch the culture condition, by changing the medium to supply. Here we evaluate how efficiently the medium in the well is exchanged. In the presence of non-motile *E. coli* W3110 ΔfliC Δflu ΔfimA , we switch the medium to supply from phosphate buffered saline (PBS) to PBS solution of rhodamine fluorescent dye, and monitor the fluorescent signal in a cross-section of the device by a confocal microscope (Supplementary Fig. 3a, from right to left). The result shows that the medium inside the well is exchanged uniformly in space (Supplementary Fig. 3b) and that it is almost completed within 2–4 min (Supplementary Fig. 3d). We also change the medium from PBS with rhodamine to that without rhodamine (Supplementary Fig. 3a, from left to right). The exchange then took longer time, ≈ 5 min, presumably because of adsorption of rhodamine on the substrate and membrane (see Supplementary Fig. 3a). In any case, the time to take for exchanging medium is much shorter than the timescale of the bacterial cell cycle. Our observations also indicate that the membrane is indeed kept flat above the well (Supplementary Fig. 3a) and that the Brownian motion of non-motile cells is hardly affected by relatively strong medium flow above the membrane (estimated at roughly 6 mm/sec) induced when switching the medium (Supplementary Movie 5,6). We therefore conclude that the EMPS is indeed able to change the growth condition for cells under observation uniformly, without noticeable fluid flow perturbations.

Characterization of bacterial reductive division by EMPS

Now we observe the reductive division of *E. coli* MG1655 in the EMPS, triggering starvation by switching from nutritious medium to non-nutritious buffer. In the beginning, a few cells are trapped in a quasi-two-dimensional well (diameter $55 \mu\text{m}$ and depth $0.8 \mu\text{m}$) and grown in nutritious medium, until a microcolony composed of approximately 100 cells appear. We then quickly switch the medium to a non-nutritious buffer, which is continuously supplied until the end of the observation (see Methods for more details). By doing so, we intend to remove various substances secreted by cells, such as autoinducers for quorum sensing and waste products, to reduce their effects on cell growth [29–32]. Throughout this experiment, the well is entirely recorded by phase contrast microscopy. We then measure the length and the width of all cells in the well, to obtain the volume v of each cell by assuming the spherocylindrical shape, at different times before and after the medium switch. Here we mainly show the results for the case where the medium is switched from LB broth to PBS (denoted by $\text{LB} \rightarrow \text{PBS}$) in Fig. 3, while the results for $\text{M9}(\text{Glc}+\text{a.a.}) \rightarrow \text{PBS}$, and $\text{M9 medium with glucose (Glc)} \rightarrow \text{M9 medium with } \alpha$ -

FIG. 3. Results from the observations of reductive division. **a** Snapshots taken during the reductive division process of *E. coli* MG1655 in the EMPS. The medium is switched from LB broth to PBS at $t = 0$. See also Supplementary Movie 7. **b,c** Experimental data (blue symbols) for the total cell volume $V_{\text{tot}}(t)$ (**b**), the growth rate $\lambda(t)$ (**b**, Inset), the mean cell volume $V(t)$ (**c**) and the number of the cells $n(t)$ (**c**, Inset) in the case of LB $\rightarrow$ PBS, compared with the simulation results (red curves). The error bars indicate segmentation uncertainty in the image analysis (see Methods). $t = 0$ is the time at which PBS enters the device (black dashed line). The data were collected from 15 wells. **d** Time evolution of the cell size distributions during starvation in the case of LB $\rightarrow$ PBS at $t = 0, 5, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480$ min from right to left. The sample size is $n(t)$ for each distribution (see **c**, Inset). **e** Rescaling of the data in (**d**). The overlapped curves indicate the function $F(v/V(t))$ in Eq. (2). The dashed line represents the time average of the datasets. **f** CV and Sk [Eq. (3)] against $V(t) = \langle v \rangle$. The error bars were estimated by the bootstrap method with 1000 realizations.

methy-D-glucoside (α MG), a glucose analog which can-282 not be metabolized [33], are also presented in Supple-283 mentary Fig. 4 and S5. We observe that, after switch-284 ing to the non-nutritious buffer, the growth of the total-285 volume decelerates (Supplementary Movie 7-9, Fig. 3b,286 Supplementary Fig. 4b and Supplementary Fig. 5b), and287 the mean cell volume rapidly decays because of exces-288 sive cell divisions (Supplementary Movie 7-9, Fig. 3a,c).289 The volume change is mostly due to the decrease of the290 cell length, while we notice that the mean cell width may291 also change slightly (Supplementary Fig. 6). We consider292 that this is not due to osmotic shock [34], because then293 the cell width would increase when the osmotic pressure294 is decreased, which is contradictory to our observation295 for LB $\rightarrow$ PBS (Supplementary Table 1 and Supplemen-296 tary Fig. 6). Such a change in cell widths was also re-297 ported for a transition between two different growth con-298 ditions [35]. In any case, Fig. 3d shows how the distribu-299 tion of the cell volumes v , $p(v, t)$, changes over time; as300 the mean volume decreases, the histograms shift leftward301 and become sharper. However, when we take the ratio302 $v/V(t)$, with $V(t) = \langle v \rangle$ being the mean cell volume at303 each time t , and plot $vp(v, t)$ instead, we find that all304 those histograms overlap onto a single curve (Fig. 3e).305 In other words, we find that the time-dependent cell size306 distribution during the reductive division maintains the307 following scale-invariant form all the time:

$$p(v, t) = v^{-1} F(v/V(t)). \quad (2)$$

This is analogous to Eq. (1) previously reported for the steady growth condition, but here importantly the mean volume $V(t)$ changes over time significantly (Fig. 3c). To further test the scale invariance of the distribution, we evaluate the coefficient of variation (CV) and the skewness (Sk) defined by

$$\text{CV} \equiv \frac{\sqrt{\langle \delta v^2 \rangle}}{\langle v \rangle}, \quad \text{Sk} \equiv \frac{\langle \delta v^3 \rangle}{\langle \delta v^2 \rangle^{3/2}}, \quad (3)$$

with $\delta v \equiv v - \langle v \rangle$. Both quantities measure the shape of the distribution function of $v/V(t)$ and not affected by variation of $V(t)$. The results in Fig. 3f indeed confirm that both CV and Sk remain essentially constant, so that the function $F(\cdot)$ remains unchanged and the scale invariance holds during the reductive division. Remarkably, we reach the same conclusion for all starving conditions that we test, as shown in Fig. 4a,b,c (see Fig. 3f, Supplementary Fig. 4d and Supplementary Fig. 5d for the results of CV and Sk) [36]. Our results therefore indicate that the scale invariance as in Eq. (2), which has been observed for steady conditions [1, 2], also holds in non-steady reductive division processes of *E. coli* rather robustly.

In addition to the robustness of the scaling relation (2), the functional form of the scale-invariant distribution, i.e., that of $F(x)$, is of central interest. We detected weak dependence of $F(x)$ on the choice of the medium in the growth phase (Fig. 4c). The lower the nutrient level of the growth medium is, the sharper the function

FIG. 4. Rescaled cell size distributions. **a** The results for M9(Glc+a.a.) → PBS. The dashed line represents the time average of the datasets. The data were taken from 17 wells. The sample size ranges from $n(0) = 685$ to $n(180) = 1260$ (see Supplementary Fig. 4c). (Inset) Time evolution of the non-rescaled cell size distributions at $t = 0, 10, 20, 30, 40, 50, 60, 90, 120, 180$ min. **b** The results for M9(Glc) → M9(αMG). The dashed line represents the time average of the datasets. The data were taken from 26 wells. The sample size ranges from $n(-5) = 1029$ to $n(65) = 1591$ (see Supplementary Fig. 5c). (Inset) Time evolution of the non-rescaled cell size distributions at $t = -5, 5, 10, 20, 25, 30, 35, 50, 65$ min. **c** Experimental results of $F(v/V(t)) = vp(v, t)$ for the three cases studied in this work. The raw data obtained at different times are shown by thin lines with relatively light colors, and the time-averaged data are shown by the bold lines. The time-averaged distributions (bold lines) are found to be slightly but significantly different among the three cases. The difference can also be seen in the instantaneous distributions (thin lines; see the inset for enlargement near the peak). **d** Fitting of the experimentally obtained $F(x)$ (solid lines; time-averaged data in (c) are shown) to the log-normal distribution (yellow dotted line). Also shown is the fitting result by Giometto *et al.* [1] for unicellular eukaryotes (green dotted line). σ is the standard deviation parameter of the log-normal distribution (see text).

$F(x)$ becomes, and therefore, the smaller the variance is. This is somewhat unexpected in view of the past studies reporting the robustness of cell size fluctuations against varying temperatures and other environmental factors [1, 5, 7] [37].

Moreover, we find that our observations for *E. coli* are significantly different from those for unicellular eukaryotes reported by Giometto *et al.* [1] (Fig. 4d). More precisely, they showed that the rescaled cell size distribution for unicellular eukaryotes is well fitted by the log-normal distribution, $\propto (1/x) \exp(-(\log x - m)^2/2\sigma^2)$ with $m = -\sigma^2/2$ (due to the normalization $\langle x \rangle = 1$), and obtained $\sigma = 0.471(3)$. We find that our data for *E. coli* can also be fitted by the log-normal distribution (Fig. 4d, yellow dotted line), but here the value of σ , evaluated by the standard deviation of $\log x$, is found to be around

$\sigma = 0.3$ ($\sigma = 0.34(1)$) for LB → PBS, $\sigma = 0.29(2)$ for M9(Glc+a.a.) → PBS, and $\sigma = 0.28(1)$ for M9(Glc) → M9(αMG), much lower than $\sigma = 0.471(3)$ for the unicellular eukaryotes. In the literature, a previous study on *B. subtilis* [38] reported values of σ from 0.24 to 0.26, which are comparable to our results for *E. coli*. Compared to this substantial difference between bacteria and unicellular eukaryotes, the dependence on the environmental factors seems to be much weaker, as suggested by our observations under three different growth conditions (Fig. 4d).

Modeling the reductive division process

To obtain theoretical insights on the experimentally observed scale invariance of the cell size distributions, we construct a simple cell cycle model for the bacterial reductive division. For the steady growth conditions, a large number of studies on *E. coli* have been carried out to clarify what aspect of cells triggers the division event [8, 9]. Significant advances have been made recently to provide molecular-level understanding [8, 19, 20, 35, 39–42]. Here we extend such a model to describe the starvation process.

One of the most established models in this context is the Cooper-Helmstetter (CH) model [18, 43], which consists of cellular volume growth and multifork DNA replication. The multifork replication is the phenomenon that a cell replicates its DNA not only for its daughters but also for its granddaughters, before the birth of the daughter cells (Fig. 5a) – a phenomenon well known for fast growing bacteria such as *E. coli* and *B. subtilis* [18, 43]. In the CH model, completion of the DNA replication triggers the cell division, and this gives a homeostatic balance between the DNA amount and the cell volume. An unknown factor of the CH model is how DNA replication is initiated, and a few studies attempted to fill this gap to complement the CH model [19, 20]. Ho and Amir [19] assumed that replication is initiated when a critical amount of “initiators” accumulate at the origin of replication. In the presence of a constant concentration of autorepressors, expressed together with the initiators, this assumption means that the cellular volume increases by a fixed amount between two initiation events, regardless of the absolute volume at the initiation. This “adder” principle between initiations is now supported by several observations [40–42]. In contrast, Wallden *et al.* [20] proposed another model based on their experimental observation. In their model, the initiation occurs when the cell volume exceeds a given threshold, independent of the volume added since the last initiation. Clarifying the mechanism of cell division control is currently a target of intensive studies and more refined models have been proposed [40–42]. However, in the present work, we choose to start from the simpler model by Ho and Amir

FIG. 5. Model of reductive division and simulation results. **a** Single (top) and multifork (bottom, where $\#ori = 4$) intracellular cycle processes. See Eq. (4) for the criterion that triggers the initiation. Progress of each cycle is represented by a coordinate $X_i^{CD}(t)$, which ends at $X_i^{CD}(t) = X_i^{CD,th}$ by triggering cell division. In the multifork process, all intracellular cycles proceed simultaneously at the same rate $\mu(t)$. **b** Illustration of cell cycles in this model. Each colored arrow represents a single intracellular cycle process. **c** Overlapping of the rescaled cell size distributions during starvation in the model for LB $\rightarrow$ PBS. The dashed line represents the time average of the datasets. (Inset) The non-rescaled cell size distributions at $t = 0, 5, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480$ min from right to left. **d** Numerically measured division rate, $B(v, t)$, in the model for LB $\rightarrow$ PBS. See Supplementary information for the detailed measurement method. (Inset) Test of the condition of Eq. (9). Here $B_t(0)/B_t(t)$ is evaluated by $B_t(0)/B_t(t) = \int B(xV(0), 0)dx / \int B(xV(t), t)dx$, with x running in the range $0 \leq x \leq 1.8$. Overlapping of the data demonstrates that Eq. (9) indeed holds in our model.

[19], which can already reproduce major characteristics of cell division control in the exponential growth phase, in particular the adder principle between birth and division [9, 44, 45]. We extend Ho and Amir's model to cope with the switch to the non-nutritious condition, and measure the cell size fluctuations during the reductive division process. We also checked that our main conclusions do not change if we extend Wallden *et al.*'s model instead. The model consists of two processes that proceed simultaneously, namely the volume growth and the intracellular cycle. The volume of each cell (indexed by i), $v_i(t)$, grows as $\frac{dv_i}{dt} = \lambda(t)v_i(t)$, with a time-dependent growth rate $\lambda(t)$. Following Ho and Amir's CH model [19], we assume that the volume growth is coupled to the intracellular cycle as follows. To begin with the simplest case, suppose that a newborn cell i has a single origin of replication in its chromosome, and that the replication starts at some point in time (Fig. 5a, top, red star). By this initiation of replication, the cell starts to have two origins of replication. Then, in Ho and Amir's model, the next initiation is triggered when the cell volume $v_i(t)$ increases by a fixed amount $\delta_{i,1}$ per origin, i.e.,

when $v_i(t)$ increases by $\delta_{i,1} \times 2$, since the last initiation (Fig. 5a, top, blue stars). Note that this criterion does not change whether a cell divides or not before the initiation; if a cell divides and produces daughter cells i_1 and i_2 , the initiation in the daughter cells occurs when $v_{i_1}(t) + v_{i_2}(t) - v_i(t_{init}) = \delta_{i,1} \times 2$, where t_{init} is the time at which the last initiation occurred. Similarly, if multifork replication takes place in a single cell (i.e., $\#ori = 2^j$ with $j \geq 2$), the threshold for the added volume is given by $\delta_{i,j} \times \#ori$ (see Fig. 5a, bottom, for an example with $\#ori = 4$). The criterion therefore reads:

$$\left(\sum_{i' \in \text{offspring of } i} v_{i'}(t) \right) - v_i(t_{init}) = \delta_{i,j} \times \#ori. \quad (4)$$

Following the experimental results by Si *et al.* [39], we assume that $\delta_{i,j}$ does not depend on environmental conditions. On the other hand, to take into account stochastic nature of division events, we generate $\delta_{i,j}$ randomly from the Gaussian distribution with mean $\langle \delta_{i,j} \rangle = \delta_{mean}$ and standard deviation $Std[\delta_{i,j}] = \delta_{std}$.

By the initiation considered above, the cell starts the

C period of the bacterial cell cycle, which is followed by the D period, and eventually the cell divides [18, 20, 43]. Here, for the sake of simplicity, all these processes are grouped together and expressed as the C+D period. Its progression is represented by a coordinate $X_i^{\text{CD}}(t)$, which starts from zero and increases at time-dependent speed $\mu(t)$, $\frac{dX_i^{\text{CD}}}{dt} = \mu(t)$. When $X_i^{\text{CD}}(t)$ reaches a threshold $X_i^{\text{CD},\text{th}}$, the cell divides (Fig. 5a, bottom), leaving two daughter cells of volumes $v_{i_1}(t) = x^{\text{sep}}v_i(t)$ and $v_{i_2}(t) = (1 - x^{\text{sep}})v_i(t)$. Here, x^{sep} is randomly drawn from the Gaussian distribution with mean 0.5 (see below and Supplementary Fig. 7 for its standard deviation). To deal with the multifork replication, the index i of $X_i^{\text{CD}}(t)$ denotes the cell to divide by the considered cell cycle progression. Therefore, if $\#ori = 2$ when the initiation is triggered, a pair of cell cycles for the future daughter cells, represented by $X_{i_1}^{\text{CD}}(t)$ and $X_{i_2}^{\text{CD}}(t)$, start and run simultaneously (Fig. 5(b)). Similarly to $\delta_{i,j}$, we also assume that $X_i^{\text{CD},\text{th}}$ is a Gaussian random variable, with $\langle X_i^{\text{CD},\text{th}} \rangle = 1$ and $\text{Std}[X_i^{\text{CD},\text{th}}] = X_{\text{std}}^{\text{CD},\text{th}}$.

Now we are left to determine the two time-dependent rates, $\lambda(t)$ and $\mu(t)$. Here we consider the situation where growth medium is switched to non-nutritious buffer at $t = 0$; therefore, t denotes time passed since the switch to the non-nutritious condition. First, we set the volume growth rate $\lambda(t)$ on the basis of the Monod equation [46], assuming that substrates in each cell are simply diluted by volume growth and consumed at a constant rate without uptake because of the non-nutritious condition considered here. As a result, we obtain

$$\lambda(t) = \lambda_0 \frac{1 - A}{e^{Ct} - A}, \quad (5)$$

with constant parameters A and C , and the growth rate $\lambda_0 (= \lambda(0))$ in the exponential growth phase (see Supplementary information for details). For the cycle progression speed $\mu(t)$, we first note that the C+D period mainly consists of DNA replication, followed by its segregation and the septum formation [43]. Most parts of those processes involve biochemical reactions of substrates, such as deoxynucleotide triphosphates for the DNA synthesis, and assembly of macromolecules such as FtsZ proteins for the septum formation. We therefore assume that the cycle progression speed is determined by the intracellular concentration of relevant substrates and macromolecules, which is known to decrease in the stationary phase [47, 48]. Specifically, we assume that the cycle progression speed is expressed by the Hill equation, $\mu(t) \propto [\text{RM}_{\text{C+D}}]^n / (K^n + [\text{RM}_{\text{C+D}}]^n)$, with $[\text{RM}_{\text{C+D}}]$ being the concentration of the relevant molecules and K, n adjustable parameters. $[\text{RM}_{\text{C+D}}]$ decreases by dilution due to volume growth, degradation, and consumption. Those are assumed not to depend on $\#ori$, based on the experimental results that the duration of the C+D period is independent of $\#ori$ in steady environments [39].

Finally, we obtain the following equation for the cycle progression speed:

$$\mu(t) = \mu_0 \frac{k + 1}{k \exp(t/\tau) + 1}, \quad (6)$$

with parameters k and τ , and the cycle progression speed $\mu_0 (= \mu(0))$ in the growth phase (see Supplementary information).

The parameter values are determined from the experimentally measured total cell volume and the cell number, which our simulations turn out to reproduce very well (Fig. 3b,c and Supplementary Fig. 4b,c), with the aid of relations reported by Wallden *et al.* [20] for some of the parameters (see Table I for the parameter values used in the simulations, and Methods for the estimation method). With the parameters fixed thereby, we measure the cell size fluctuations at different times and find the scale invariance similar to that revealed experimentally (Fig. 5c and Supplementary Fig. 8b,d). The constancy of CV and Sk is also confirmed (Supplementary Fig. 8a,c). We also evaluate the functional form of the numerically obtained distribution through the fitting to the log-normal distribution, and obtain $\sigma = 0.24(1)$. This value is comparable to the experimental one, though no information on the distribution is used in the parameter adjustment. Interestingly, the scale invariance emerges despite the existence of characteristic scales in the model definition, such as the typical volume added between initiations, δ_{mean} . To check the robustness of those results, we also extended Wallden *et al.*'s model along the same line and confirmed the scale invariance of similar quality (Supplementary Fig. 8e,f) (see Supplementary information for details of the model). These findings suggest the existence of a statistical principle underlying the scale invariance, which is not influenced by details of the model.

Theoretical conditions for the scale invariance

To seek for a possible mechanism leading to the scale invariance, here we describe, theoretically, the time dependence of the cell size distribution in a time-dependent process. Suppose $N(v, t)dv$ is the number of the cells whose volume is larger than v and smaller than $v + dv$. If we assume, for simplicity, that a cell of volume v can divide to two cells of volume $v/2$, at probability $B(v, t)$, we obtain the following time evolution equation:

$$\begin{aligned} \frac{\partial N(v, t)}{\partial t} = & - \frac{\partial}{\partial v} [\lambda(t)vN(v, t)] \\ & - B(v, t)N(v, t) + 4B(2v, t)N(2v, t). \end{aligned} \quad (7)$$

Note that this equation has been studied by numerous past studies for understanding stable distributions in steady conditions [1, 45, 49–53], but here we explicitly

include the time dependence of the division rate, $B(v, t)$, for describing the transient dynamics. To clarify a condition for this equation to have a scale-invariant solution, here we assume the scale invariant form, Eq. (2), where $p(v, t) = N(v, t)/n(t)$ and $n(t)$ is the total number of the cells, and obtain the following self-consistent equation (see Supplementary information for derivation):

$$F(x) = -x \frac{\partial F(x)}{\partial x} - \frac{B(v, t)}{\bar{B}(t)} F(x) + 2 \frac{B(2v, t)}{\bar{B}(t)} F(2x). \quad (8)$$

Here, $x = v/V(t)$ and $\bar{B}(t) = \int dv B(v, t) p(v, t)$. For the scale invariance, Eq. (8) should hold at any time t . This is fulfilled if $B(v, t)$ can be expressed in the following form (see Supplementary information):

$$B(v, t) = B_v(v/V(t)) B_t(t). \quad (9)$$

This is a sufficient condition for the cell size distribution to maintain the scale invariant form, Eq. (2), during the reductive division. It is important to remark that, as opposed to Eq. (7), Eq. (8) does not include the growth rate $\lambda(t)$ explicitly. The scale-invariant distribution $F(x)$ is therefore completely characterized by the division rate $B(v, t)$ in this framework.

To test whether the condition of Eq. (9) is satisfied in our model, we measure the division rate $B(v, t)$ in our simulations (Fig. 5d). The data overlap if $B(v, t) B_t(0)/B_t(t)$ is plotted against $v/V(t)$, demonstrating that Eq. (9) indeed holds here. In our model, the cycle progression speed $\mu(t)$ is assumed to be given completely by the concentration of cell cycle-related substrates, which takes the same value for all cells. This may be why the separation of variable, Eq. (9), effectively holds, and the scale invariance follows. On the other hand, our theory does not seem to account for the functional form $F(x)$ of the scale-invariant distribution; the right hand side of Eq. (8) differs significantly from the left hand side, if the numerically obtained $B(v, t)$ is used together with the function $F(x)$ from the simulations or the experiments (Supplementary Fig. 9). The disagreement did not improve by taking into account the effect of septum fluctuations (see Supplementary information). The lack of quantitative precision is probably not surprising given the simplicity of the theoretical description, which incorporates all effects of intracellular cycles into the simple division rate function $B(v, t)$. The virtue of this theory is that it clarifies that the intracellular cycle seems to have important relevance in the scale invariance and the functional form of the cell size distribution. The significant difference in $F(x)$ identified between bacteria and unicellular eukaryotes (Fig. 4d) may be originated from the different replication mechanisms that the two taxonomic domains adopt.

Violation of the scale invariance

Here we investigate the robustness of the scale invariance during the reductive division. In particular, we aim to clarify whether it breaks down for other starvation conditions, and if so, what the condition is for the scale invariance to hold. As shown in Fig. 4c, the form of $F(x)$ obtained by our experiments depends on the growth environment before starvation. This suggests that $F(x)$ may change if one switches between two growth media in a quasistatic manner, i.e., the scale invariance may break down in this case. Motivated by this hypothesis, we numerically investigate whether there is a lower bound on the relaxation speed of the cellular state, below which the scale invariance breaks down. For simplicity, we consider that the environment starts to change at $t = 0$, and the volume growth rate $\lambda(t)$ and the cycle progression speed $\mu(t)$ decrease as follows:

$$\lambda(t) = \lambda(0) \exp(-t/\tau_\lambda), \quad (10)$$

$$\mu(t) = \mu(0) \exp(-t/\tau_\mu). \quad (11)$$

We regard τ_λ , τ_μ and $\lambda(0)$ as free parameters, while $\mu(0)$ is determined from $\lambda(0)$, using empirical relation reported by Wallden *et al.* [20] for steady environments. The number of cells is set to be approximately 50,000 at $t = 0$ and kept constant afterward, by eliminating one of the daughter cells produced by division (see Methods for details).

First we evaluate the mean cell volume $\langle v \rangle$ and the coefficient of variation, $CV = \sqrt{\langle \delta v^2 \rangle} / \langle v \rangle$, in the exponential growth phase under steady conditions, by varying the growth rate $\lambda(0)$ from 0.0075 to 0.03 /min. As shown by the black squares in Fig. 6a,b, lower growth rates (smaller mean volumes) lead to lower CVs. This is consistent with our experimental results (Fig. 4c). We then investigate how the mean volume $\langle v \rangle$ and CV change during the starvation process, starting at $t = 0$ from the growth phase with $\lambda(0) = 0.03$ /min (Fig. 6a, the top right black square). As expected, our data showed that the mean cell volume decreases if $\tau_\mu > \tau_\lambda$ and increases otherwise; therefore, in the following, we deal with the case of $\tau_\mu > \tau_\lambda$, which corresponds to the reductive division. The color curves in Fig. 6 show trajectories in the $\langle v \rangle$ -CV space during the starvation process, each curve corresponding to a different $\tau_\mu (> \tau_\lambda)$ with τ_λ fixed at $\tau_\lambda = 40$ min. Remarkably, these trajectories overlap to a single curve with an extended plateau region, which indicates that CV is kept constant, i.e., the scale invariance. Each curve stops in the middle of the master curve, the location of the endpoint (at $t \rightarrow \infty$, shown by the open circles) being determined by τ_μ . Importantly, for small τ_μ , the trajectories stop in the plateau region, so that the scale invariance holds during the entire process. By contrast, for large τ_μ , the trajectories go over the plateau and CV decreases abruptly; in other words the scale invari-

FIG. 6. Numerical results on the range of validity of the scale invariance. The initial growth rate is fixed at $\lambda(0) = 0.03$ /min unless otherwise stipulated. **a** Trajectories in the $\langle v \rangle$ -CV space for different τ_μ (from 50 to 150 min), with $\tau_\lambda = 40$ min fixed. The endpoint of each trajectory is indicated by a colored open circle. The black squares represent the states in steady growth conditions with the growth rate $\lambda(0)$ ranging from 0.0075 to 0.03 /min. **b** The master curves of the $\langle v \rangle$ -CV trajectories for different τ_λ . Those are obtained by taking the average of the CV values at each $\langle v \rangle$ over different $\tau_\mu (> \tau_\lambda)$. τ_λ ranges from 10 to 120 min. **c** Phase diagram. $\times$: the scale invariance breaks down. Blue $\circ$: the scale invariance holds. Green $\triangle$: near the boundary. Black dots: the scale invariance holds but the mean volume increases. **d** Pseudocolor plot of ρ for different τ_λ and τ_μ . See the main text for the definition of ρ . The black region indicates $\rho = 0$. The white line represents the transition line obtained from (c). The boundaries ($\triangle$) are included in the region where the scale invariance breaks down.

ance breaks down. Next, Fig. 6b shows the master curves for different τ_λ , each constructed by using the trajectories for all τ_μ greater than τ_λ . We find that the smaller τ_λ is, the more extended the plateau region is. Finally, we show the phase diagram for various combinations of τ_μ and τ_λ in Fig. 6c. This clearly shows a region in which the scale invariance is maintained during the entire starvation process, bordered by a transition line over which the scale invariance breaks down. Note that the scale-invariant region becomes narrower for larger τ_λ and τ_μ and seem to disappear eventually; this is consistent with our expectation described at the beginning, that the scale invariance does not hold for quasistatic changes. All those results are also confirmed for another initial condition, prepared with $\lambda(0) = 0.015$ /min (Supplementary Fig. 10).

To understand what triggers the violation of the scale invariance, we focus on the state of the multifork replications, since our theory suggested the importance of the division rate, which is controlled by the state of the cell cycle. First, we observe that the change in the number of origins of replication ($\#ori$) during the growth phase is rather stable, doubling (by initiation) and decreasing

(by division) between $\#ori = 2^{j-1}$ and 2^j with a fixed j for the majority of cells ($j = 3$ in the case of Fig. 5b; note that $j - 1$ and j correspond to the numbers of parallel arrows therein). This number is maintained for a while in the starvation process, but eventually it may decrease, because a cell may divide without initiating a new replication during the life. We therefore measure the fraction of such cells, ρ . To be precise, with J being the number of j of the majority of cells in the growth phase, ρ is the fraction of cells such that the C+D period with $j < J$ is initiated during the lifetime, and that this C+D period ends and triggers a cell division afterward, before the cell cycle progression completely stops (note that, since $\mu(t) \rightarrow 0$, not all cell cycles complete). It is measured at the final time point of the simulations (specifically $t = 600$ min). Figure 6d shows the results for $\lambda(0) = 0.03$ /min, for which $J = 3$ in the growth phase. Here we find $\rho = 0$ indeed in most part of the scale-invariant region, while $\rho > 0$ when the scale invariance breaks down. We therefore conclude that the state of the multifork replications is indeed a key factor that determines whether the scale invariance holds or not during the reductive division. Note that, for gradual environmental changes, actual cells are known to emit signals such as ppGpp [54, 55] that control growth and cycle progression, which are not taken into account in our model. Investigating the effect of such signals in this problem is an interesting problem left for future studies.

DISCUSSION

In this work, we developed a novel membrane-based microfluidic device that we named the extensive microperfusion system (EMPS), which can realize a uniformly controlled environment for wide-area observations of microbes. We believe that the EMPS has potential applications in a wide range of problems with dense cellular populations, including living active matter systems [56, 57] and biofilm growth [58–60]. Here we focused on statistical characterizations of single cell morphology during the reductive division of *E. coli*. Thanks to the EMPS, we recorded the time-dependent distribution of cell size fluctuations and revealed that the rescaled distribution is scale-invariant and robust against the abrupt environmental change, despite the decrease of the mean cell size. This finding was successfully reproduced by simulations of a model based on the CH model [19, 20], which we propose as an extension for dealing with time-dependent environments. We further inspected theoretical mechanism behind this scale invariance and found the significance of the division rate function $B(v, t)$. We obtained a sufficient condition for the scale invariance, Eq. (9), which was indeed confirmed in our numerical data. Finally, we numerically clarified the range of validity of the scale invariance during the reductive division, revealing

the crucial role played by the state of the multifork repli-
cations. Notably, our experiment (the culture condition
dependence, Fig. 4c) and simulations (Fig. 6c) suggest
that the scale invariance breaks down for slow starva-
tion. Further investigations of cell size fluctuations in
such cases, both experimentally and theoretically, will
be an important step toward clarifying what determines
the critical time scale of environmental changes for the
violation of the scale invariance.

After all, our theory suggests that mechanism of intra-
cellular replication processes may have direct impact on
the scale-invariant distribution, which may account for
the significant difference we identified between bacteria
and eukaryotes (Fig. 4d). Since the number of species
studied in each taxonomic domain is rather limited (*E. coli*
(this work) and *B. subtilis* [38] for bacteria, 13 pro-
tist species for eukaryotes [1]), it is of crucial impor-
tance to test the distribution trend further in each tax-
onomic domain, and to clarify how and to what extent
the cell size distribution is determined by the intracellu-
lar replication dynamics. Combining with other theoret-
ical methods, such as models considering the cellular age
[61], knowledge from the universal protein number fluctu-
ations [62, 63], and renormalization group approaches for
living cell tissues [64], may be useful in this context. The
influence of cell-to-cell interactions, e.g., quorum sensing
[29, 30], may also be important. We hope that our under-
standing of the population-level response against nutrient
starvation will be further refined by future experimental
and theoretical investigations.

METHODS

Strains and culture media

We used wild-type *E. coli* strains (MG1655 and RP437)
and a mutant strain (W3110 Δ flhC Δ flhA Δ flhB) in this
study. Culture media and buffer are listed in Supplemen-
tary Table 1. The osmotic pressure of each medium was
measured by the freezing-point depression method by the
OSMOMAT 030 (Genotec, Berlin Germany). Details on
the strains and culture conditions in each experiment are
provided below.

Fabrication of the EMPS

The EMPS consists of a microfabricated glass cov-
erslip, a bilayer porous membrane and a PDMS pad.
The microfabricated coverslip and the PDMS pad were
prepared according to ref. [21, 23]. We fabricated
the bilayer porous membrane by combining a strepta-
vidin decorated cellulose membrane and a biotin deco-
rated polyethylene-terephthalate (PET) membrane. The

streptavidin decoration of the cellulose membrane (Spec-
tra/Por 7, Repligen, Waltham Massachusetts, molecu-
lar weight cut-off 25000) was realized by the method de-
scribed in ref. [21, 23]. The PET membrane (Transwell
3450, Corning, Corning New York, nominal pore size
0.4 μ m) was decorated with biotin as follows. We soaked
a PET membrane in 1 wt% solution of 3-(2-aminoethyl
aminopropyl) trimethoxysilane (Shinetsu Kagaku Kogyo,
Tokyo Japan) for 45 min, dried it at 125°C for 25 min
and washed it by ultrasonic cleaning in Milli-Q water for
5 min. This preprocessed PET membrane was stored in
a desiccator at room temperature, until it was used to
assemble the EMPS.

The EMPS was assembled as follows. The prepro-
cessed PET membrane was cut into 5 mm $\times$ 5 mm squares,
soaked in the biotin solution for 4 hours and dried on fil-
ter paper. The biotin decorated PET membrane was at-
tached with a streptavidin decorated cellulose membrane,
cut to the size of the PET membrane, by sandwich-
ing them between agar pads (M9 medium with 2 wt%
agarose). In the meantime, a 1 μ l droplet of bacterial
suspension was inoculated on a biotin decorated cover-
slip (see also details below). We then took the bilayer
membrane from the agar pad, air-dried for tens of sec-
onds, and carefully put on the coverslip on top of the
bacterial suspension. The bilayer membrane was then
attached to the coverslip via streptavidin-biotin binding
as shown in Supplementary Fig. 1b. We then air-dried
the membrane for a minute and attached a PDMS pad
on the coverslip by a double-sided tape.

Cell growth measurement in U-shape traps in the PDMS-based device

We used a non-motile mutant strain W3110 without
flagella and pili (Δ flhC Δ flhA Δ flhB) to prevent cell ad-
hesion to the surface of a coverslip. Before the time-
lapse observation, we inoculated the strain from a glyce-
rol stock into 2 ml M9 medium with glucose and amino
acids (Glc+a.a.) (see also Supplementary Table 1) in a
test tube. After shaking it overnight at 37 °C, we trans-
ferred 20 μ l of the incubated suspension to 2 ml fresh
M9(Glc+a.a.) medium and cultured it until the OD at
600 nm wavelength reached 0.4-0.5. We then injected
the bacterial suspension into the device from the cell in-
let (Supplementary Fig. 2b) and left it until a few cells
entered the U-shape traps. The device was placed on
the microscope stage, in the incubation box maintained
at 37 °C. The microscope we used was Leica DMI8,
equipped with a 63x (N.A. 1.30) oil immersion objective
and operated by Leica LasX.

During the observation, we constantly supplied
M9(Glc+a.a.) medium and 0.5 wt% bovine serum
albumin (BSA) from the medium inlet (Supplemen-
tary Fig. 2b) at the rate of 0.7 ml/hr (flow speed in the

drain was approximately 1 mm/sec). Cells were observed by phase contrast microscopy and recorded at the time interval of 3 min. The velocity field of the coherent flow was obtained by particle image velocimetry, using MatPIV (MATLAB toolbox). The stream-wise component of the velocity field (Fig. 2(c)) was averaged over the span-wise direction, and also over the time period of 150 min.

Cell growth measurement in U-shape traps in the EMPS

Here the choice of the strain, the medium, the culture condition and the instruments was the same as those for the measurement with the PDMS-based device, unless otherwise stipulated. Here, the cultured bacterial suspension was diluted to OD = 0.04 before it was inoculated on the coverslip of the EMPS. As sketched in Fig. 2(a), the substrate consisted of drain channels (100 μ m wide, 7 mm long, 13 μ m deep) and U-shape traps (30 μ m wide, 80 μ m long, 1.0 μ m deep), which were prepared by the methods described in ref. [23]. When the bilayer membrane was attached to the substrate, care was taken not to cover the two ends of the drain channel to use, so that cells in the drain could escape from it. After the assembly of the device with the bacterial suspension, it was fixed on the microscope stage inside the incubation box maintained at 37 °C. To fill the device with medium, we injected fresh medium stored at 37 °C from the inlet (Supplementary Fig. 1), at the rate of 60 ml/hr for 5 min by a syringe pump (NE-1000, New Era Pump Systems). During the observation, the flow rate of the M9(Glc+a.a. and BSA) medium was set to be 2 ml/hr (approximately 0.2 mm/sec above the membrane), except that it was increased to 60 ml/hr (approximately 6 mm/sec above the membrane) at a constant interval in order to remove the cells expelled from the trap efficiently. The time interval of this flush was 60 min before the observed trap was filled with cells, and 30 min thereafter.

Observation of the bacterial reductive division

We used a wild-type strain MG1655. Before the time-lapse observation, we inoculated the strain from a glycerol stock into 2 ml growth medium in a test tube. The same medium as for the main observation was used (LB broth, M9(Glc+a.a.) or M9(Glc)). After shaking it overnight at 37 °C, we transferred 20 μ l of the incubated suspension to 2 ml fresh medium and cultured it until the OD at 600 nm wavelength reached 0.1-0.5. The bacterial suspension was finally diluted to OD = 0.05 before it was inoculated on the coverslip.

For this experiment, we used a substrate with wells of 55 μ m diameter and 0.8 μ m depth. The well di-

ameter was chosen so that all cells in the well can be recorded. The device was placed on the microscope stage, in the incubation box maintained at 37 °C. The microscope we used was Leica DMI8, equipped with a 100x (N.A. 1.30) oil immersion objective and operated by Leica LasX. To fill the device with growth medium, we injected fresh medium stored at 37 °C from the inlet (Supplementary Fig. 1), at the rate of 60 ml/hr for 5 min by a syringe pump (NE-1000, New Era Pump Systems).

In the beginning of the observation, growth medium was constantly supplied at the rate of 2 ml/hr (flow speed approximately 0.2 mm/sec above the membrane). When a microcolony composed of approximately 100 cells appeared, we quickly switched the medium to a non-nutritious buffer (PBS or M9 medium with α -methyl-D-glucoside (α MG), see Supplementary Table 1) stored at 37 °C, by exchanging the syringe. The flow rate was set to be 60 ml/hr for the first 5 minutes, then returned to 2 ml/hr. Throughout the experiment, the device and the media were always in the microscope incubation box, maintained at 37 °C. Cells were observed by phase contrast microscopy and recorded at the time interval of 5 min.

The cell volumes were evaluated as follows. We determined the major axis and the minor axis of each cell, manually, by using a painting software. By measuring the axis lengths, we obtained the set of the lengths L and the widths w for all cells. We then estimated the volume v of each cell, by assuming the spherocylindrical shape of the cell: $v = \frac{4\pi}{3} (\frac{w}{2})^3 + \pi (\frac{w}{2})^2 (L - w)$. We estimated the uncertainty in manual segmentation at $\pm 0.15 \mu$ m.

Simulation

The parameters used in the simulations were evaluated as follows. First, from the observations of the exponential growth phase, we determined the growth rate λ_0 directly. This allowed us to set the cycle progression speed μ_0 too, by using the relation $\mu_0^{-1} \simeq (1.3\lambda_0^{-0.84} + 42)$ proposed by Wallden *et al.* [20] (the values of λ_0 and μ_0 in the unit of min^{-1} are used here). Concerning the volume threshold for initiating the replication, we found such a value of δ_{mean} (or $v_{\text{mean}}^{\text{th}}$) that reproduced the experimentally observed mean cell volume in the growth phase. The standard deviation δ_{std} (or $v_{\text{std}}^{\text{th}}$) was set to be 10% of the mean δ_{mean} ($v_{\text{mean}}^{\text{th}}$), based on the relation on the initiation volume found by Wallden *et al.* [20]. They also measured the fluctuations of the time length of the C+D period; this led us to estimate $X_{\text{std}}^{\text{CD,th}}$ at 5% of $\langle X^{\text{CD,th}} \rangle$, i.e., $X_{\text{std}}^{\text{CD,th}} = 0.05$. On the septum positions, we measured their fluctuations and found little difference in $x_{\text{std}}^{\text{sep}}$ among the different growth conditions we used, and also in the non-nutritious case (Supplementary Fig. 7). We therefore used a single value $x_{\text{std}}^{\text{sep}} = 0.0325$ for all simulations.

TABLE I. Parameters used for the simulations.

Parameters	Parameters	Base: Ho and Amir [19]		Base: Wallden <i>et al.</i> [20]	
		LB→PBS	M9(Glc+a.a.)→PBS	LB→PBS	LB→PBS
Parameters on the exponential growth phase	$\lambda(0) = \lambda_0$	0.029 /min	0.010 /min	0.029 /min	0.029 /min
	$\mu(0)^{-1} = \mu_0^{-1}$	$1.3\lambda_0^{-0.84} + 42 \simeq 67$ min	$1.3\lambda_0^{-0.84} + 42 \simeq 104$ min	$1.3\lambda_0^{-0.84} + 42 \simeq 67$ min	$1.3\lambda_0^{-0.84} + 42 \simeq 67$ min
	δ_{mean} or $v_{\text{mean}}^{\text{th}}$	$\delta_{\text{mean}} = 0.25 \mu\text{m}^3$	$\delta_{\text{mean}} = 0.225 \mu\text{m}^3$	$v_{\text{mean}}^{\text{th}} = 1.0 \mu\text{m}^3$	$v_{\text{mean}}^{\text{th}} = 1.0 \mu\text{m}^3$
	δ_{std} or $v_{\text{std}}^{\text{th}}$	$0.1 \times \delta_{\text{mean}} = 0.025 \mu\text{m}^3$	$0.1 \times \delta_{\text{mean}} = 0.0225 \mu\text{m}^3$	$0.1 \times v_{\text{mean}}^{\text{th}} = 0.1 \mu\text{m}^3$	$0.1 \times v_{\text{mean}}^{\text{th}} = 0.1 \mu\text{m}^3$
	$X_{\text{std}}^{\text{CD,th}}$	$0.05 \times \langle X_i^{\text{CD,th}} \rangle = 0.05$	$0.05 \times \langle X_i^{\text{CD,th}} \rangle = 0.05$	$0.05 \times \langle X_i^{\text{CD,th}} \rangle = 0.05$	$0.05 \times \langle X_i^{\text{CD,th}} \rangle = 0.05$
	$x_{\text{std}}^{\text{sep}}$	0.0325	0.0325	0.0325	0.0325
Time-dependent rates	$\lambda(t) = \lambda_0 \frac{1-A}{e^{Ct}-A}$	$A = 0.93, C = 0.0059$ /min	$A = 0.84, C = 0.011$ /min	$A = 0.93, C = 0.0059$ /min	$A = 0.93, C = 0.0059$ /min
	$\mu(t) = \mu_0 \frac{k+1}{ke^{t/\tau}+1}$	$k = 1, \tau = 60$ min	$k = 0.1, \tau = 24$ min	$k = 10, \tau = 75$ min	$k = 10, \tau = 75$ min

In the following, we describe how the remaining parameters were evaluated and how the simulations were carried out for each set of the simulations presented in this work.

tally obtained population $n_{\text{exp}}(t)$ (Fig. 3c and Supplementary Fig. 4c), the numerically obtained population $n_{\text{sim}}(t)$ is rescaled by multiplying $n_{\text{exp}}(0)/n_{\text{sim}}(0)$.

Methods for the results that reproduced the experimental observations

We evaluated the time-dependent rates $\lambda(t)$ and $\mu(t)$ as follows. The growth rate $\lambda(t)$ can be determined independently of the cell divisions, because the total volume $V_{\text{tot}}(t) = \sum_i v_i(t)$ grows as $V_{\text{tot}}(t) = V_{\text{tot}}(0) \exp(\int_0^t \lambda(t) dt)$. With $\lambda(t)$ given by Eq. (5), we compared $V_{\text{tot}}(t)$ with experimental data and determined the values of A and C (Fig. 4c). Finally, only k and τ in Eq. (6) remained as free parameters. We tuned them so that the mean cell volume $V(t)$ and the number of cells $n(t)$ observed in the simulations reproduced those from the experiments (Fig. 4d).

The parameter values determined thereby are summarized in Table I, for the simulations for LS→PBS and M9(Glc+a.a.)→PBS. Note that we did not perform simulations for M9(Glc)→M9(α MG), because we found it difficult to fit the experimental data for $V_{\text{tot}}(t)$ and $n(t)$ in this case (Supplementary Fig. 5). This suggests that the consequence of the replacement of glucose by glucose analog α MG may not be simple starvation.

We started the simulations from 10 cells with volumes in the range of 0.07-1.13 μm^3 , randomly generated from the uniform distribution. The cells grew in the exponential phase (with the constant growth rate λ_0 and the cycle progression speed μ_0) until the number of cells reached 100,000. We then randomly picked up 10 cells from this “precultured” sample and grew them until the number of cells exceeded 500. Those cells were kept growing for 1000 minutes to sufficiently mix cell cycle progressions in the population. During this process, we kept the number of cells constant, by eliminating one of the daughter cells after each division. We then used them as the initial population of each simulation. To precisely compare the number of cells in simulations with the experimen-

Methods for the results on violation of the scale invariance

The functional forms of $\lambda(t)$ and $\mu(t)$ were given by Eqs. (10) and (11), with variable parameters τ_λ and τ_μ . The other parameters were fixed at $\delta_{\text{mean}} = 0.25 \mu\text{m}^3$, $\delta_{\text{std}} = 0.025 \mu\text{m}^3$, $X_{\text{std}}^{\text{CD,th}} = 0.05$, and $x_{\text{std}}^{\text{sep}} = 0.0325$. We started the simulations from 50 cells with volumes in the range of 0.07-1.13 μm^3 , randomly generated from the uniform distribution. The cells grew in the exponential phase until the number of cells reached 500,000. We then randomly picked up 50 cells and grew them until the number of cells exceeded 50,000. Those cells were kept growing for 1,000 minutes to sufficiently mix cell cycle progressions in the population, with the number of cells kept constant by eliminating one of the daughter cells produced by division. Using them as the initial population (at $t = 0$), we started the simulations for $t \geq 0$, with the number of cells still kept constant by the same method.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS

T.S. and K.A.T. designed research. T.S., R.O., Y.W. and K.A.T. developed the extensive microperfusion system. T.S. performed all bacterial experiments and analyzed data. T.S. and K.A.T. did the modeling, and T.S. wrote the codes for the simulations. T.S. performed the theoretical calculations. T.S. and K.A.T. wrote the manuscript, and all authors worked for revision.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CODE AVAILABILITY

The codes used in this study are available from the corresponding author upon request.

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Figures

Figure 1

Sketch of the extensive microperfusion system (EMPS). a Entire view of the device. Microwells are created on a glass coverslip. We attach a PDMS pad on the coverslip with a square frame seal to ll the system with liquid medium. b Cross-sectional view inside the PDMS pad. A PET-cellulose bilayer porous membrane is attached via the biotin-streptavidin bonding.

Figure 2

Cell growth measurements in U-shape traps in EMPS. **a** Sketch of the design of microchannels. Non-motile cells are trapped in the shallow observation area. Cells in the trap can escape to the deep drain channel. **b** Top view of the trap ($30 \mu\text{m} \times 80 \mu\text{m}$, $1:0 \mu\text{m}$ depth) filled with *E. coli* W3110 Δ ic Δ u Δ mA (see also Supplementary Movie 4). The scale bar is $25 \mu\text{m}$. Coherent flow of cells driven by cell proliferation is directed toward the drain ($15 \mu\text{m}$ depth). **c** The stream-wise (y) component of the velocity field averaged over the span-wise (x) direction (the two-dimensional velocity field is shown in Supplementary Fig. 2c), $u(y) = \langle u(x; y) \rangle_x$, measured in different time periods. The data were taken from a single trap. $t = 0$ is the time at which the trap is filled with cells. Error bars represent the standard error, which we roughly evaluate by picking up 5 data points along the spanwise direction, at intervals of roughly $5 \mu\text{m}$, half of the size of the PIV window. The open end is located near $y \approx 81 \mu\text{m}$. (Inset) The same quantity measured for a PDMS-based device with a similar trap (Supplementary Fig. 2a,b,c). The open end is at $y \approx 88 \mu\text{m}$. Time dependence is clearly observed. **d** The growth rate profiles λ , evaluated by $\lambda(y) = \langle \frac{1}{h} \frac{du(x; y)}{dy} \rangle_x$, for the EMPS (main panel) and the PDMS-based device (inset). Error bars represent the standard error of the ensemble.

Figure 3

Results from the observations of reductive division. a Snapshots taken during the reductive division process of *E. coli* MG1655 in the EMPS. The medium is switched from LB broth to PBS at $t = 0$. See also Supplementary Movie 7. b,c Experimental data (blue symbols) for the total cell volume $V_{\text{tot}}(t)$ (b), the growth rate $\lambda(t)$ (b, Inset), the mean cell volume $V(t)$ (c) and the number of the cells $n(t)$ (c, Inset) in the case of LB ! PBS, compared with the simulation results (red curves). The error bars indicate segmentation uncertainty in the image analysis (see Methods). $t = 0$ is the time at which PBS enters the device (black dashed line). The data were collected from 15 wells. d Time evolution of the cell size distributions during starvation in the case of LB ! PBS at $t = 0$; 5; 30; 60; 90; 120; 180; 240; 300; 360; 420; 480 min from right to left. The sample size is $n(t)$ for each distribution (see c, Inset). e Rescaling of the data in (d). The overlapped curves indicate the function $F(v=V(t))$ in Eq. (2). The dashed line represents the time average of the datasets. f CV and Sk [Eq. (3)] against $V(t) = \langle v \rangle$. The error bars were estimated by the bootstrap method with 1000 realizations.

Figure 4

Rescaled cell size distributions. a The results for M9(Glc+a.a.) → PBS. The dashed line represents the time average of the datasets. The data were taken from 17 wells. The sample size ranges from $n(0) = 685$ to $n(180) = 1260$ (see Supplementary Fig. 4c). (Inset) Time evolution of the non-rescaled cell size distributions at $t = 0; 10; 20; 30; 40; 50; 60; 90; 120; 180$ min. b The results for M9(Glc) → M9(αMG). The dashed line represents the time average of the datasets. The data were taken from 26 wells. The sample size ranges from $n(5) = 1029$ to $n(65) = 1591$ (see Supplementary Fig. 5c). (Inset) Time evolution of the non-rescaled cell size distributions at $t = 5; 10; 20; 25; 30; 35; 50; 65$ min. c Experimental results of $F(v=V(t)) = vp(v, t)$ for the three cases studied in this work. The raw data obtained at different times are shown by thin lines with relatively light colors, and the time-averaged data are shown by the bold lines. The time-averaged distributions (bold lines) are found to be slightly but significantly different among the three cases. The difference can also be seen in the instantaneous distributions (thin lines; see the inset for enlargement near the peak). d Fitting of the experimentally obtained $F(x)$ (solid lines; time-averaged data in (c) are shown) to the log-normal distribution (yellow dotted line). Also shown is the fitting result by Giometto et al. [1] for unicellular eukaryotes (green dotted line). σ is the standard deviation parameter of the log-normal distribution (see text).

Figure 5

Model of reductive division and simulation results. **a** Single (top) and multifork (bottom, where $\#ori = 4$) intracellular cycle processes. See Eq. (4) for the criterion that triggers the initiation. Progress of each cycle is represented by a coordinate $X_{CD}^i(t)$, which ends at $X_{CD}^i(t) = X_{CD,th}^i$ by triggering cell division. In the multifork process, all intracellular cycles proceed simultaneously at the same rate $\dot{X}(t)$. **b** Illustration of cell cycles in this model. Each colored arrow represents a single intracellular cycle process. **c** Overlapping of the rescaled cell size distributions during starvation in the model for LB $\rightarrow$ PBS. The dashed line represents the time average of the datasets. (Inset) The non-rescaled cell size distributions at $t = 0; 5; 30; 60; 90; 120; 180; 240; 300; 360; 420; 480$ min from right to left. **d** Numerically measured division rate, $B(v, t)$, in the model for LB $\rightarrow$ PBS. See Supplementary information for the detailed measurement method. (Inset) Test of the condition of Eq. (9). Here $B_t(0) = B_t(t)$ is evaluated by $B_t(0) = B_t(t) = \int_0^1 B(xV(0); 0) dx = \int_0^1 B(xV(t); t) dx$, with x running in the range $0 \leq x \leq 1/8$. Overlapping of the data demonstrates that Eq. (9) indeed holds in our model.

Figure 6

Numerical results on the range of validity of the scale invariance. The initial growth rate is fixed at $\lambda(0) = 0.03$ /min unless otherwise stipulated. a Trajectories in the hvi-CV space for different τ_μ (from 50 to 150 min), with $\tau_\lambda = 40$ min fixed. The endpoint of each trajectory is indicated by a colored open circle. The black squares represent the states in steady growth conditions with the growth rate $\lambda(0)$ ranging from 0.0075 to 0.03 /min. b The master curves of the hvi-CV trajectories for different τ_λ . Those are obtained by taking the average of the CV values at each hvi over different τ_μ ($\tau_\mu > \tau_\lambda$). τ_λ ranges from 10 to 120 min. c Phase diagram. $\times$: the scale invariance breaks down. $\circ$: the scale invariance holds. $\triangle$: near the boundary. Black dots: the scale invariance holds but the mean volume increases. d Pseudocolor plot of ρ for different τ_μ and τ_λ . See the main text for the definition of ρ . The black region indicates $\rho = 0$. The white line represents the transition line obtained from (c). The boundaries (4) are included in the region where the scale invariance breaks down.

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