Step by Step, Cell by Cell: Quantification of the Bacterial Cell Cycle

Cell cycle, step by step, cell by cell

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Abstract

The *Escherichia coli* cell cycle is a classic, but we are still missing some of its essential aspects. The reason is that our knowledge is mostly based on population data, and our grasp of the behavior of single cells is still very limited. Today, new dynamic single-cell data promise to overcome this barrier. Existing data from single cells already led to findings and hypotheses that challenge standard views, and opened questions that did not yet settle. Here, we review these recent developments and propose that a systematic exploration of the correlation patterns between cell cycle intervals defined by key molecular events measured in many single cells could lead to a quantitative characterization of the cell cycle as an interplay of stochastic and homeostatic events.

Keywords: Cell-cycle, Division Homeostasis, E. coli, Stochasticity, Key intervals, Replication initiation

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1. The cell cycle is a feature of single cells.

We might think that we know a great deal about the cell cycle of *E. coli*, and this is, to some extent, true. Certainly, the subject does not strike as new. The discovery of a key role of **replication initiation** (see Glossary) dates back to work from the late 1960s [1, 2], and already in the early 1990s the reviews arguably listed most of the molecular players that we consider relevant today [3].

However, it is not difficult to convince ourselves that we are missing some essential aspects of the problem. The reason is that most of the information in our possession comes from bulk **population measurements** and indirect inference. In this Opinion piece, we would like to argue that today it is the right moment to revisit the problem exploting dynamic **single-cell measurements**. Such experiments require efficient imaging and cell segmentation-tracking methods, and are helped by microfluidic control of nutrient exchange. Used in combination with reporters of molecular events and protein expression, they may build a new basis for understanding the unraveling of the cell cycle. This data will be accessible in the coming years. And we are in for a few surprises.

We focus here on *E. coli* as a model organism, where, e.g. one can build on the large existing molecular biology knowledge, but the range of applicability of this approach can be broad, extending to the cell cycle control in eukaryotic species, where the same problem of identifying key determinants emerges [4, 5, 6].

2. The average cell is not the typical single cell

From the pioneers of quantitative bacterial physiology of the "Copenhagen School" [7], empirical observations in terms of quantitative relations between physiology-related variables averaged over large populations have been used to infer specific control mechanisms of the cell cycle [8, 7]. The problem is that the average cell behaviour does not correspond necessarily to the typical behaviour of single cells. Therefore, models based on population averages have limitations, and must be revisited and tested with single-cell data. The classic example is the model proposed by Donachie [2] in the 60s, stating that DNA replication is initiated at a critical mass per replication origin. As we will discuss, although appealing and perfectly compatible with results from bulk growth, this model looks incompatible with recent single-cell measurements, supporting the assertion that multiple beliefs on the cell cycle ought to be re-studied on single cells. In other words, there are unique specific behaviors of single live cells that are obscured if we average everything (something to be careful about even when studying single cells).

For the cell cycle to progress, events related to DNA replication and **segregation**, metabolism, growth and cell division must occur in a specific time order for each cell, across many divisions, i.e., along lineages of cells [9, 10], despite considerable molecular noise and variability of parameters. As an example of this hierarchy and its long-term impact, a missed **septation** due to late segregation may lead to failure of cell division, formation of a filamentous cell and subsequent rescue, which can be accompanied by a non-symmetric division, with consequences on the balance of cell size, DNA amounts and cell-cycle regulators observable over several generations. However, this chain

of events could affect a small fraction of the population, and thus it could be clearly observable at the level of bulk growth only in severe and unrealistically stressful conditions. Therefore, dissecting such a cascade of errors and controls would be impossible without a single-cell view, since the complex temporal interplay of several concurrent processes and the important role of **stochasticity** are hidden by population averages

3. From phenomenological to mechanistic models

A clear sign that the application of high-throughput single-cell techniques is effective comes from recent work, which has helped characterizing the growth-division cycle. Such measurements have already unveiled the stochastic nature of metabolism and resulting growth [11], intriguing universal properties of the joint distribution of cell size and interdivision times [12, 13], as well as an effective principle where cells add, on average, a constant volume to their initial one every cell division (sometimes called **adder mechanism**) [14, 12, 15], which is consistent with long-term **homeostasis** of the cell-size distribution in a population. However, how the adder mechanism comes about molecularly remains unknown. Indeed, several phenomenological models can in principle reproduce the empirical observation of a constant average added size. For example, a "concerted" control of cell division based on cell size and on time [16, 13] as well as a completely different mechanism based on the ratio between cell surface and volume [17] can both reproduce this behaviour. Therefore, it is necessary to link more closely these available minimal phenomenological models to molecular mechanism in order to discriminate between the direct molecular controls and indirect results (which

may come, e.g., from physiological constraints or hidden optimization principles).

4. Cell cycle intervals

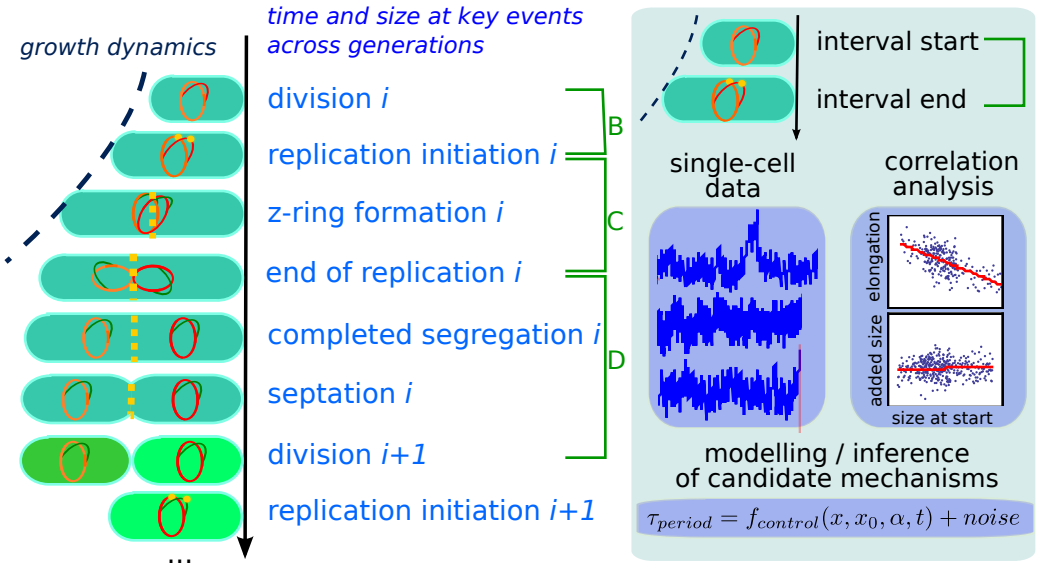

Figure 1: Key events in the *E. coli* cell cycle define cell-cycle intervals subject to stochastic variation and exerting homeostatic control. The drawing illustrates some of the key cellular events (replication cycle, Z ring formation, septation, segregation, etc.) which may be used to define intervals. Such events are stochastic due to intrinsic molecular noise and to cell-to-cell variability of cellular parameters. Homeostatic control can be exerted if completion of the interval is correlated with important events. Time-hierarchy (and eventually causality) between events can be inferred by accumulating a large statistics of cells and consecutive generations. Cell-cycle intervals may not span just the time between two cell divisions, but can be defined across consecutive generations. Correlation analysis and mathematical modeling can help linking intervals and molecular players to homeostasis of key parameters, such as cell size and cell content.

To move our mathematical descriptions towards more specific biological

mechanisms, a first step is to focus the analysis on specific cell cycle events that have been directly linked to molecular controls. Indeed, a common way to describe qualitatively the progression of the cell cycle [1, 18, 19] is to define **cell-cycle intervals** (Fig. 1), by key landmark events (e.g., replication, formation of the Z-ring, septation, etc.), and establishing their relative timing and connection with global observables such as cell growth rate, size, total protein concentration, as well as with concentration of selected metabolic or cell-cycle proteins (e.g., a reliable reporter of the initiator protein DnaA [20, 21]).

Note that cell cycle intervals may span multiple consecutive generations, and are not necessarily defined within two consecutive divisions. For example, it might make sense to consider the events of completion of a successful segregation and onset of septation in one cycle, and link them to the event of replication initiation in the following one, or consider the period between termination and initiation (typically in the next generation), where ATP-DnaA is supposed to increase [22, 23]. More trivially, it is well known that the timing of replication, the "C period", can be longer than the interdivision time [1, 24], since fast-growing *E. coli* cells support multiple DNA replication rounds at the same time, and thus the replication initiation in one cell cycle will lead to a complete chromosome in a subsequent cycle of a daughter or grand-daughter cell. Reporters of at least some of the key players of the cell-cycle are at hand, thanks to many previous studies characterizing several aspects of the E. coli cell cycle [25, 26, 27].

Each measured interval may effect decisions, meaning that its completion is conditional to some measurable parameters (say, cell size or growth rate),

but at the same time carries sources of errors due to molecular noise and cell-to-cell variability in key parameters such as concentrations of regulators or metabolic enzymes. Cells adjust cell-cycle intervals to respond to specific needs (e.g. conditioning division to successful nucleoid segregation), creating a structure of statistical correlations and conditional dependencies between interval durations and measurable parameters. These trends allow to detect both the (statistical) time hierarchy of cell-cycle events, and possible homeostatic controls effected during one interval. They also enable the production of testable quantitative mathematical descriptions of the cell cycle.

Note that this simplification needs to be handled with care. For example intervals might be hard to define for chromosome segregation, which is a multi-step process [28]. Equally, reporters of expression of cell-cycle proteins do not automatically define intervals, but they may be used to define them by their oscillations, spatial organization, or threshold values. More in general, protein expression and spatial distributions can be correlated with cell cycle progression along defined cell-cycle intervals.

5. Studying single cells can challenge long-standing hypotheses.

A number of recent studies have produced data that are already challenging existing models, and highlight the importance of further investigations [30, 19, 29]. One example is the "licensing hypothesis" for replication initiation [19], which, based on observations on single cells, proposes that septation or occurrence of cell division may license (by activating the origin or releasing an inhibitory signal) the chromosomes for the next round of replication initiation (and unlicensed origins cannot initiate).

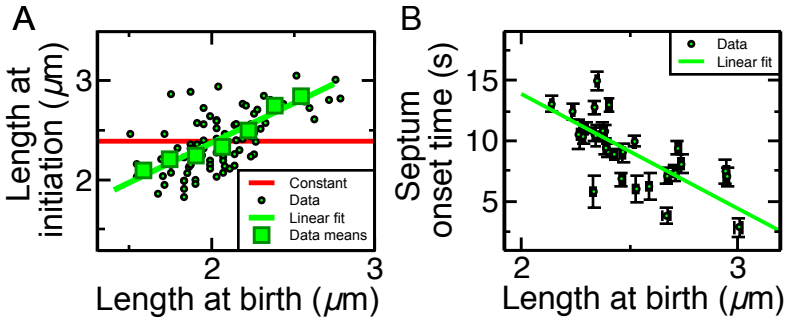

Figure 2: Examples of insights from correlation analysis of cell-cycle intervals. A: Available data for replication initiation in single cells contradict the hypothesis of initiation at a critical cell size (mass). The plot (data from ref. [29]) shows the cell size at replication initiation (estimated by the appearance of SeqA-GFP foci) versus the initial cell size (estimated by cell length, cell width being constant across cells). Each circle corresponds to a different cell cycle. A critical mass model (red line) would predict that the initiation size is the same regardless of size at birth. Instead, while there is some control on size, cells tend to be larger at initiation if they are born larger (green, squares are binned averages of data, solid line is a linear fit). B: Septum onset time enforces size homeostasis. The plot (data from ref.[30]) shows the septum onset time (measured by cell shape segmentation[31]) versus the initial cell size. A pure characteristic septation time would show no correlation with length at birth, but the existence of a correlation suggests a correction mechanism. A recent study on perturbations of volume and surface growth rates supports this observation [17].

Concerning the timing of replication initiation and cell division [29], Donachie [2], based on population data [32, 1], proposed that DNA replication is initiated at a critical mass per replication origin. Notably, this author was very aware that the critical mass hypothesis is only a sufficient condition to comply to the constraints imposed by the behavior of population averages. Other mechanisms than a critical size (mass) at initiation are compatible with the same constraints on population averages. The lack of a precise critical initiation size may be consistent with models in which the initiation time is set by the relative levels of **DnaA** bound to ADP or to ATP [25, 22]. In fact, theoretical descriptions that do not comply with the critical mass hypothesis are present in the literature. For example, a recent modeling study [33] argues that initiation may occur after a constant size has been added between consecutive initiations.

Fluorescent labeling of replication forks has been used to start adresssing these questions in single cells [29, 34]. These studies indicate that the timing of initiation is indeed dependent on birth size, i.e., cells that are born larger than average initiate earlier. This supports a role for replication initiation in maintaining size homeostasis. A similar correlation with size size was observed for the D-period between termination and division [29]. Whether size compensation at initiation is due to a perfect critical size remains incompletely resolved. One study found indications that cells that are born larger than average initiate at sizes that are slightly larger than mean size at initiation across the population (Fig. 2), while the data of another study was found to be consistent with a constant initiation volume model [34], attributing the observed correlation in Fig. 2A to the constraint that the initation size

should be larger than the initial cell size. In comparing different studies, one must also consider possible effects of labelling schemes: one can for instance label all SeqA proteins [34], or only a fraction of them [29], or use different labels of the replication fork, such as DnaQ [34]. In this case, the data of these two studies appear consistent between them and susceptible to both interpretations, leaving the question open to new tests and measurements.

A second example of useful information from cell-cycle intervals at the single-cell level is the hypothesis of a role of septum formation in homeostasis. Single-cell analysis (Fig. 2) indicates that the cell cycle interval from cell birth to onset of septation (measured by cell segmentation) may be size-dependent (and hence may effect homeostasis) [30]. More recent and more extended results [17] have lead to speculate that septum formation may be the main ("rate limiting") checkpoint in deciding cell division in most conditions. Conversely, the cell cycle interval defined by the timing between onset of septation and cell division fluctuates around a constant value, independent of the total interdivision time, much like the C period [30].

In conclusion, these studies illustrate the gap of knowledge on the cell cycle at the single-cell level, provide first answers, and indicate the potential of correlating events and processes in single cells.

6. How do cell-cycle intervals add up to produce size homeostasis and cell cycle control?

Deeper knowledge of the most relevant cell-cycle intervals, reflecting key processes such as replication cycle and the triggering of Z-ring contraction, will help answering how different controls exerted during the cell cycle con-

tribute to achieving size homeostasis, a constant added size, and scaling properties of cell sizes and interdivision times. Clearly, from the biological viewpoint, characterizing the cell cycle is a broader aim than mere characterization of cell-size homeostasis, but understanding the link between cell-size distribution, metabolism and key molecular determinants may have important implications. Taking the example of the observed constant added size [12, 14], one may link this behavior to a classic "initiator" model [35] where the key step (replication initiation) is triggered by the accumulation of an initiator protein to a constant copy number (not concentration), which is compatible with the observation that the total amount of active DnaA appears to be relevant for initiation timing in *E. coli* [25]. However, several processes may contribute to the decision to divide. Besides the process of replication initiation by DnaA [22, 23], the division triggering of the Z-ring [18, 36], conditioned on successful segregation [37], as well as metabolic cues [26, 38] and septum synthesis [17] have all been linked to cell division.

Analysis of the concerted action of these control mechanisms should show whether the decision to divide is based on a single "rate limiting" principle, whether different controls may be rate limiting in different conditions, or whether controls are active on overlapping time scales. Incidentally, none of the intervals defined by DNA replication appear to obey constant added size in slow-growing cells, but a model tuned on these cell cycle intervals does reproduce the overall constant added size behavior [29].

A (complementary) possibility to explore is that observations such as the constant added size or the scaling of size and doubling time fluctuations could be the result of external constraints, such as optimization principles (e.g.,

for global colony growth or lineage expansion) or avoidance of detrimental effects (e.g., waste accumulation) acting on top of the integration of cues from different sources leading to cell division. These constraints may be found in ecology [39, 13], where ubiquitous scaling laws linking e.g. cell (body) size and metabolism have been observed.

7. Concluding remarks and future perspectives.

Perhaps the most important feature of the investigation we propose is that it is fully quantitative. The current challenge is to produce quantitative measurement of the key players and their statistics, with the ultimate goal of summarizing them in a mathematical equations capturing all observed behaviors, able to predict phenotypes at the single-cell level. This theoretical description will need to be predictive, as well as incorporating the sources of variability across cells and the sources of error correction, linked with the key molecular players.

A quantification of cell cycle intervals in single cells can be complemented by their change in response to the external conditions, mutations, and other perturbations such as arresting replication, depleting DnaA, expressing unnecessary proteins, etc. [17, 40, 41]. For example, nutrient shifts were used classically to look at cell division dynamics [42, 43], but potentially can give a wealth of further information with contemporary techniques. A further challenge will be to understand adaptation behavior in non-steady conditions and linking this dynamic behavior to the homeostatic strengths observed in fixed environments.

Finally, focusing on cell cycle intervals that are closely linked to molecu-

lar mechanisms would give us a minimal but fully mechanistic description of the available data. Importantly, there are limitations. First, the mathematical/modeling tools for linking correlation analysis to quantitative models still need to be fully developed. Second, correlation does not necessarily reveal causality. However, we believe that such road, combined with molecular biology and biochemistry, will bring us closer to a mechanism, in comparison to the descriptions available today, which lack almost any insight into the key molecular players [15, 14, 12, 16].

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Outstanding Questions Box

What are the key molecular events in the cell cycle of single *E. coli* cells and how do they compromise or promote homeostasis of basic parameters such as cell size, protein concentration, and DNA copy number?

How are metabolic signals and housekeeping events (replication, segregation, etc.) integrated to decide when to divide?

Does the observed constant added size mechanism emerge from the integration of multiple decisions or is it the result of a single process?

TRENDS BOX

[900 characters / 3-5 bullet points]

The cell cycle is stochastic due to intrinsic cellular noise, affecting decision-making related to key steps (initiation of replication, chromosome segregation, Z-ring contraction, septation ...)

Recent high-throughput single-cell measurements of growing *E. coli* show a constant average added size between consecutive cell divisions.

Similar measurements allowing the full stochastic unraveling of the *E. coli* cell cycle will likely become available in the coming years.

These data will open new perspectives and challenge classic views, starting from the long-standing hypothesis that a critical mass per origin triggers replication initiation.

GLOSSARY

Adder mechanism: The hypothesized mechanism by which *E. coli* cells tend to add a constant volume or mass to the initial size to decide the moment of cell division. This mechanism enforces size homeostasis [14, 12]

Cell-cycle interval: Defined here as the period of time between two key events in the cell cycle (Fig. 1). For example, three cell-cycle intervals are classically defined with respect to DNA replication: the B,C,D sperated by replication initiation and the end of replication.

DnaA: ATP-ase protein that accumulates in its active ATP-bound form to a threshold value during the cell cycle inducing DNA melting by binding cooperatively to the origin(s) and thus triggering initiation of DNA replication [22].

Homeostasis: The process through which single cells control key variables (such as size, concentrations) in order to ensure their stability along lineages. There is, in general, a difference between homeostasis in fixed conditions and the average response to a perturbation.

Population measurements: Measurements of average quantities over large cell populations. Most of growth-related laws in bacterial physiology are based on such measurements [8], typically for exponentially growing populations. For example, the typical population estimate of the average cell size consists in a measurement of optical density divided by a cell count [7].

Replication initiation: The start of DNA replication, defining the end of the B period in bacteria, and corresponding to the G1/S transition in mammalian cells.

Segregation: The process of disentanglement and separation of dupli-

cated chromosomes necessary to ensure a chromosome copy to each daughter cell.

Septation: Formation of a cell wall that constricts the cell (approximately in the middle for symmetrically dividing bacteria like *E. coli*) and leads to new cell poles.

Single-cell measurements: Experiments following dynamically many cells with single-cell resolution, monitoring size, shape and fluorescent probes, and allowing to quantify the cell-to-cell variability and correlations.

Stochasticity: In the context of cell cycle events, represents the tendency of cell-cycle progression to be different in each individual cell, due to values of internal variables (e.g. key protein amounts or concentrations) and molecular noise. As a consequence, mathematical models have to describe the cell-cycle progression as a stochastic process, typically representing the interplay of cell-to-cell variability and homeostatic control.

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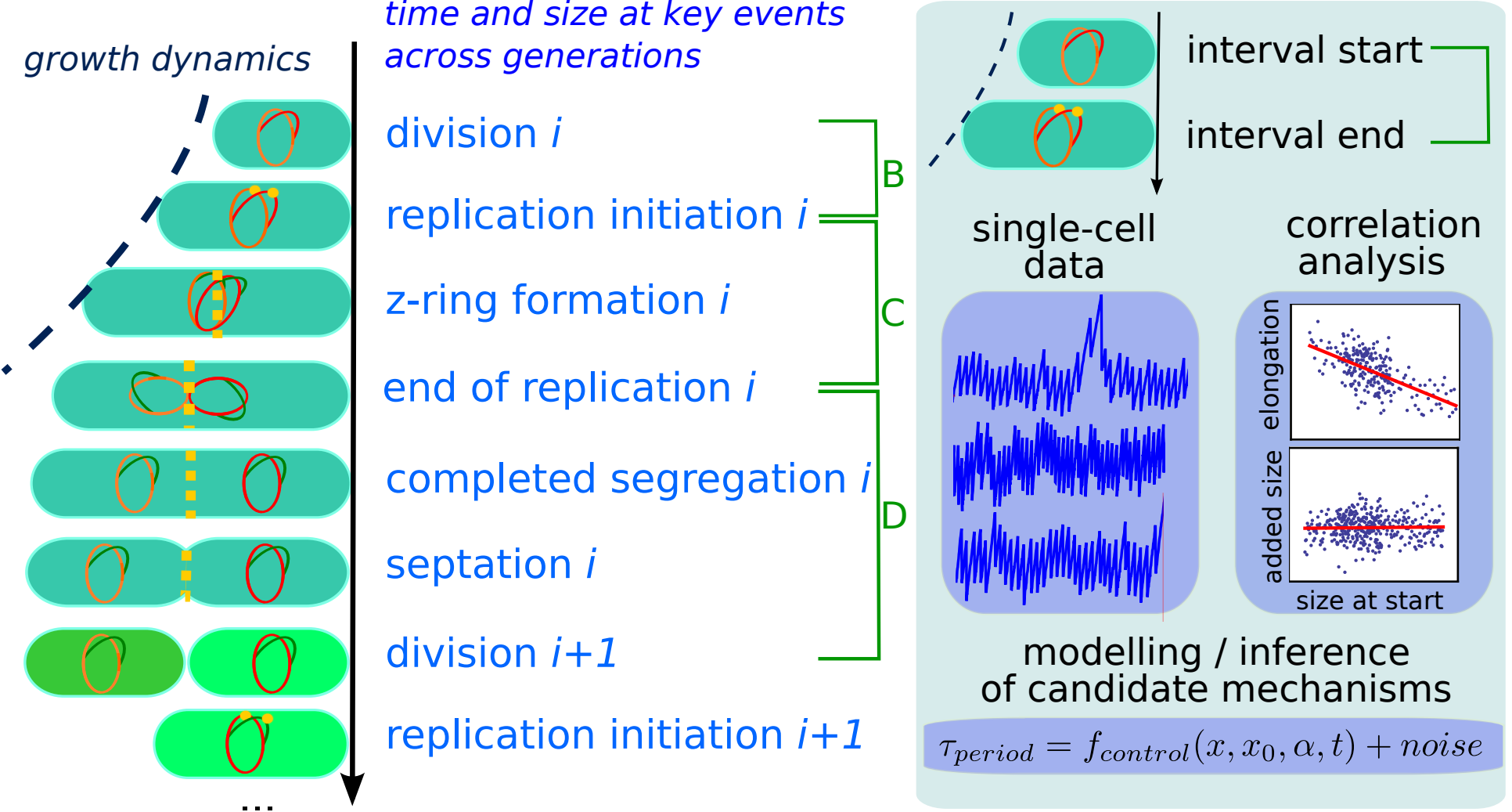
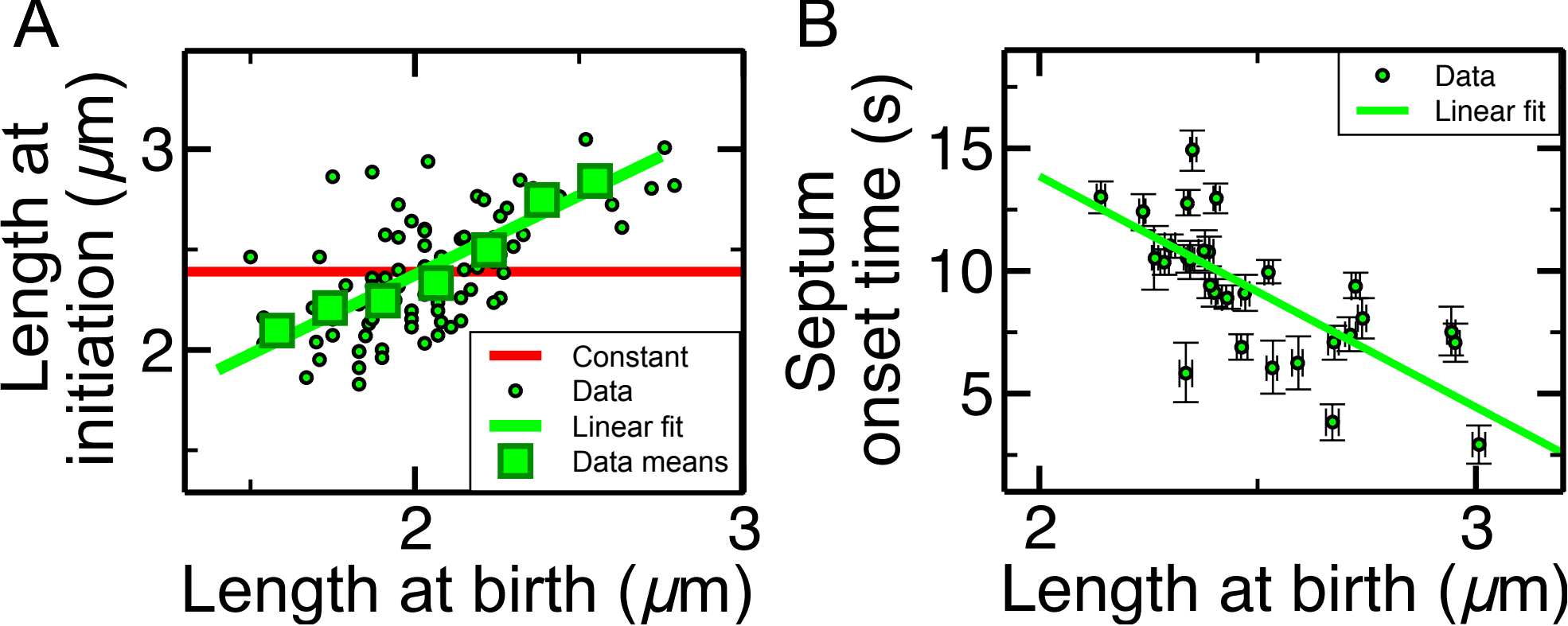
Outstanding Questions Box

- What are the key molecular events in the cell cycle of single E. coli cells and how do they compromise or promote homeostasis of basic parameters such as cell size, protein concentration, and DNA copy number?

- How are metabolic signals and housekeeping events (replication, segregation, etc.) integrated to decide when to divide?

- Does the observed constant added size mechanism emerge from the integration of multiple decisions or is it the result of a single process?


growth dynamics
time and size at key events across generations
division i
replication initiation i
z-ring formation i
end of replication i
completed segregation i
septation i
division i+1
replication initiation i+1
B
C
D
...
interval start
interval end
single-cell data
correlation analysis
elongation
added size
size at start
modelling / inference of candidate mechanisms
$\tau_{period} = f_{control}(x, x_0, \alpha, t) + noise$


A
Length at initiation (μm)
3
2
2
3
Length at birth (μm)
Constant
Data
Linear fit
Data means
B
Septum onset time (s)
15
10
5
2
3
Length at birth (μm)
Data
Linear fit

Trends Box

- The cell cycle is stochastic due to intrinsic cellular noise, affecting decision-making related to key steps (initiation of replication, chromosome segrega-tion, Z-ring contraction, septation ...)

- Recent high-throughput single-cell measurements of growing *E. coli* show a constant average added size between consecutive cell divisions.

- Similar measurements allowing the full stochastic unraveling of the *E. coli* cell cycle will likely become available in the coming years.

- These data will open new perspectives and challenge classic views, starting from the long-standing hypothesis that a critical mass per origin triggers replication initiation.