High-resolution studies of lysis–lysogeny decision-making in bacteriophage lambda

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Cellular decision-making guides complex development such as cell differentiation and disease progression. Much of our knowledge about decision-making is derived from simple models, such as bacteriophage lambda infection, in which lambda chooses between the vegetative lytic fate and the dormant lysogenic fate. This paradigmatic system is clearly understood but lacking mechanistic details, partly due to limited resolution of past studies. Here, we discuss how modern technologies have enabled high-resolution examination of lambda decision-making to provide new insights and exciting possibilities in studying this classical system. The advent of techniques for labeling specific DNA, RNA, and proteins in cells allows for molecular-level characterization of events in lambda development. These capabilities yield both new and new questions regarding how the isolated lambda genetic circuit acts, what biological events transpire among phages in their natural context, and how the synergy of simple phage macromolecules brings about complex behaviors.

Buried beneath the surface of cells is a largely uncharacterized world of biological, chemical, and physical interplay between biomolecules giving rise to life processes. As technology advances, scientists create better tools to delve into previously studied, yet still-mysterious systems to reveal the details of life with continually increasing clarity. For example, modern technologies are transforming the study of one of the oldest, best-studied model systems, bacteriophage lambda. For decades, lambda has served as a paradigm for studying gene-regulatory networks, general recombination, cellular decision-making, and other fundamental biological processes (1–3).

Lambda infects Escherichia coli and then decides between the contrasting lytic and lysogenic lifestyles. The gene regulatory network processing this decision has been well-characterized at the ensemble level (4), but there is still considerable unpredictability or “noise” associated with predicting cell fates (5). This suggests that there could be uncharacterized factors that contribute to the decision-making process, which might be addressed through proper experimentation. Before the widespread utilization of single-cell techniques to study this system, it was already clear from population-level studies that deterministic factors played an important role in the lysis–lysogeny decision. For example, increased multiplicity of infection (MOI),2 or the number of infecting phages per cell, promotes lysogeny; cell starvation (6–8) and cell size were identified as critically affecting the decision (9). These studies suggest that the characterization of more preexisting factors upon infection can reduce the observed noisiness of lambda decision-making and established a blueprint to direct future research to “explain away” more stochasticity in decision-making.

To address the remaining factors, unique perspectives and powerful tools can open a window into the cell to extract molecular details and expose hidden behaviors underlying lambda decision-making. To this end, there are a wide variety of methods to label specific DNA, RNA, and proteins for high-resolution studies. Crucially, scientists are inventing techniques to simultaneously target multiple processes, because system-wide development arises from the interactions of many players. By characterizing the individual elements of the whole and their interplay, valuable quantitative data are generated, which feed into mathematical models to achieve systems-level understanding of biology. With regard to lambda decision-making, there are open questions about its precise behavior in its native context. For example, how do lambda genetic circuits act in the cell to process decisions, what interactions occur among lambda circuits within the cell during decision-making, and what behaviors of lambda macromolecules might underpin phage development? Targeting these curiosities requires the ability to query individual viruses in single cells. In this minireview, we discuss how researchers use high-resolution techniques to probe fundamental cellular processes and how these approaches help achieve a more comprehensive understanding of lambda decision-making.

The lambda gene regulatory network

The lambda gene regulatory network has been studied for decades (see Ref. 4 for a detailed review). Recently, the lambda gene expression profile has also been investigated systematically (10). Briefly, after the ejection of phage DNA into the cytoplasm, critical decision-making genes are expressed from

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2 The abbreviations used are: MOI, multiplicity of infection; FP, fluorescent protein; FISH, fluorescent in situ hybridization.
promoters pR and pL. The pL transcript encodes N, a transcriptional anti-terminator required for expression of most early lambda genes, and CIII, a positive regulator of lysogeny. The pR transcript encodes for Cro, CII, O, P, and Q, and the organization is shown in Fig. 1. Cro serves as a negative feedback regulator for pR activity. CII is expressed after Cro and is the major mRNA levels, researchers found that cI expression is sensitive to network copy number variations resulting from DNA replication (20). Experimentation and mathematical simulations further revealed that the robustness of cII expression to DNA replication is due to Cro negative feedback on pR.

Decision-making network responses to DNA replication

The outcomes of cellular decision-making often appear noisy. The environment that the cell resides in may fluctuate, triggering different cellular responses. At the molecular level, the series of biochemical events involved in decision-making is inherently noisy. It is important to note that “noise” does not equate to pure randomness, as molecules have definite numbers and positions, but rather that current tools do not capture their exact properties, resulting in imprecise measurements of the cellular state. Noise pervades all domains of life, prompting certain questions. How is noise at the individual gene expression level propagated through intricate biological networks to exert its effects on the terminal outcomes? More specifically, how do living organisms control or take advantage of noise to perform tasks such as cellular decision-making? For phage lambda, noise utilization for phenotypic diversification has been demonstrated mathematically (18), suggesting that phages can take advantage of fluctuations in protein production rate to bifurcate into distinct pathways. Following that, a series of works have promoted the understanding of how noise can beget fate bifurcation (19). Notably, those works often focus on the mutual regulation of CI and Cro. With recent experimental evidence that CI might not be involved in the actual decision-making process, but rather to enforce and maintain the lysogenic choice (12), more mathematical models have been developed based on CII-Q competition. Moreover, other phage processes occur, but have rarely been investigated quantitatively. For example, DNA replication causes the number of intracellular phage circuits to increase exponentially, which might lead to complex changes in the overall levels of transcription and translation, yet its effects are not well-characterized.

Recently, a high-resolution study was carried out to understand the effect of DNA replication on phage decision-making, utilizing a P− phage mutant, unable to replicate its DNA (20). Interestingly, P− phages could not lyse cells, and when a single P− phage infected a cell, lysogeny did not occur either. This indicates that a single copy of the lambda genetic circuit lacks the capacity to establish either lytic or lysogenic pathways. During the early decision-making stage, the lysogenic pathway is initiated when CII accumulates and activates expression of CI from the pRE promoter. In the later decision-executing stage, CI is expressed from both the pRE and pRM promoters to establish and maintain the lysogenic state. Q serves as a “gatekeeper” for the lytic pathway. Once Q reaches a threshold, expression of the lysis and morphogenesis genes begins (11, 12). Because CI and Cro can compete for shared binding sites at the tripartite oL and oR operators to regulate each other’s expression, much focus has been placed on studying how their mutual regulation can lead to lytic–lysogenic switches (13–16). However, for the development of CI expression during infection, recent work has suggested that CII-Q competition might be the actual key for decision-making, whereas CI allows the cell to commit to the lysogenic pathway (12, 17). Simultaneously expressed from pR are O and P, essential for phage DNA replication. By introducing more copies of the gene network, DNA replication might alter the overall decision behavior.
Strategies such as feedback loops are often utilized by gene regulatory networks to control the gene expression noise and ensure more deterministic outcomes, as robustness is important for many cellular processes (20, 21). Negative feedback is a common type of regulation in gene networks. It can function to reduce noise (22–24), linearize gene expression to inputs (23, 25), and increase the frequency of fluctuations to minimize the effects of fluctuations on downstream processes, as a “slow” downstream process can only sense a time-averaged signal (26). Additionally, in the native lambda context, negative feedback loops apparently play an important role in keeping the expression of early decision genes robust to the rapidly increasing network dosages (20). In fact, multiple network features such as incoherent feed forward loops have been shown to exhibit copy number invariant gene expression (27–29), and these features often have a combination of activation and repression functions in contrast to the simple negative feedback motif observed in phage lambda. As Cro performs more functions than simple negative feedback regulation in the native lambda context (4), detailed dissection of this system is required to assess the mechanism and advantages of having robust CII expression.

Although cells evolved means to attenuate noise to achieve reliable outcomes, they can also take advantage of noise to allow a level of randomness in outputs to differentiate into different pathways. Positive feedback is a commonly used mechanism for cells with bistable switches (30–33). In a network with positive feedback, gene expression noise can be amplified to fractionate the cells into distinct subpopulations, where one group can show high expression of certain genes whereas another shows low expression. Positive feedback is also utilized by lambda in the decision-executing stage by CI. Despite phages having similar CII expression levels with or without replication, CI expression is negligible without extra copies of DNA from replication (20). Whether the positive feedback of CI from PRM is required for “all or nothing” CI expression in response to copy number changes remains to be studied.

**Phage voting for decisions**

A major advantage of high-resolution study is the ability to capture detailed individual events making up the ensemble. Through genetic engineering, fluorescent proteins (FPs) can be used to produce lambda particles with fluorescent capsids (5, 34, 35). Using this tool, the MOI can be quantified by simply counting infecting phages on each cell. By combining these fluorescent phages with a lysogenic reporter, a bold new model for decision-making was proposed, identifying the unit of decision-making as the individual infecting phage rather than the whole cell, as was assumed. In this model, infecting phages could each “vote,” or commit separately to decisions in single cells as if possessing “individuality” (5, 36). Surprisingly, these previously uncharacterized subcellular behaviors of lambda can exert critical effects on decision-making. This idea marked a profound shift in our preconceptions about viruses, where we were now required to think about the hidden complexity of viral behaviors. Following this perspective, it was reported that both lytic and lysogenic choices could proceed simultaneously during infections, resulting in “lyso-lysis,” where prophages were integrated into cells prior to lysis (37). In this study, phage behaviors were dissected at the single-DNA level using fluorescent reporters tracking phage and *E. coli* DNA in vivo in real time. Methylated phage DNA was tracked by the binding of SeqA-FP, and *E. coli* DNA was labeled at the attB (the lambda DNA integration site) locus with an inserted tetO array being bound by TetR-FP. Combined with cell-fate reporter systems, lambda DNA integration indicative of the lysogenic pathway was observed for lytic cells (37). This suggests that subcellular phage entities show a degree of individuality and can behave differently from each other. New questions emerged from these results about how the concept of individuality pertains to viruses.

**Intracellular phage interplay**

Essential to exploring phage individuality are tools that give “voices” to different phages. To achieve this, researchers adapted different lytic and lysogenic reporters into two phages (a four-color system) to allow co-infecting phages in one cell to express distinguishable lytic and lysogenic votes (38). Related to individuality is interaction, as there is a social aspect among individuals, and these tools also allowed for the exploration of phage interplay. Interestingly, lambda phages were found to compete and cooperate inside cells, indicating that different strands of DNA have different intracellular behaviors. Cooperation was found to prevail during lysogeny, where DNAs of different phages were frequently integrated into the host genome. During the lytic development, phages commonly dominated each other, resulting in only a single phage type showing lytic gene expression. Utilizing these single-cell data, computational models were constructed, consequently predicting that variables such as infection timing and host resources contributed to the behaviors, potentially informing future studies. These disparate behaviors under alternative life trajectories might be interpreted as strategic (39). Domination during lysis might be a mechanism to proliferate maximally while environmental conditions are favorable, such as in the abundance of fast-growing host cells. Conversely, cooperation may benefit phages in conditions that favor lysogeny, such as high MOI and/or cell starvation. These conditions ostensibly inform phages of host availability, so the possibility of lysogenizing multiple phages amplifies genetic drift to increase diversity in the absence of selection for lytic development.

Another interaction occurs when intracellular phages disagree in lytic/lysogenic votes, which was designated as confusion (38). This interplay typically resulted in overriding lytic votes, but in some cells, lysogeny overtook lysis. Individuality is emphasized during confusion, because it was apparent that the identity of the phages is not limited to the infecting phage. In addition to multiple phages infecting and subsequently voting for different decisions, a single infecting phage can also become confused. This is likely due to phage DNA replication, which introduces new phage individuals into the cell, suggesting that individuality is a trait belonging to the single phage genome. Fundamentally, lambda is just DNA that propagates via production of other macromolecules, so what might underlie these observed complex phenomena? The answers will be found by pushing the limits of resolution and considering underexplored perspectives in future studies.
Variability in the timing of phage infection

The decisions enacted by lambda are the end points of infection, so the kinetics of the intervening biological events contribute to decision-making. Infection begins when phage DNA enters the cell. In vitro DNA ejection experiments measured the rate at which lambda DNA leaves the virion when triggered by LamB exposure (40–42), showing that lambda DNA ejection is a fast process, around 1.5 s (40). Later, another single-cell study followed DNA ejection in vivo by staining phage DNA with a fluorescent dye and subsequently timing the transfer of fluorescent signal from the infecting phage particle into the host cell (43). The whole process was shown to average 5 min (43). Great variability in the ejection kinetics was also observed, where DNA translocation may finish in one step without interruptions or pause during the process for up to 5 min (43). In contrast to the cell-free conditions in the in vitro studies (40–42), it is reasonable that the natural barriers of the cell, the bacterial membranes and cell wall, would cause a slower and more variable ejection process in vivo. Similarly, using the SeqA-FP fusion to label phage DNA in vivo, researchers found that the appearance time of phage DNA varies in a population of infected cells, confirming that DNA ejection is an asynchronous process (34). The question then arises, how might this variability contribute to the noisiness of phage decision-making?

There are many steps during lambda development, culminating in the final decisions. Interactions between subcellular phages occur in single cells, and phage arrival timing might affect these behaviors. Intuitively, if a tardy phage were to superinfect a developing cell, it may be dominated by an early phage during lysis, as was revealed by tracking the development of different co-infecting phages (38). In lysogenic cells, there seems to be a lack of domination by the first phage, and the explanation behind this involves the transactivation of the late phage’s lysogenic reporter by the committed early phage (38). This does not mean that timing is unimportant for lysogeny, as another study showed that the later a superinfection occurs, the less the late phage contributes to the decision-making (17). Differences in the DNA arrival time can cause variations in both the timing and level of gene expression from each infecting phage, leading to distinct decision-making behaviors. Additionally, another study suggested that lysis-promoting gene expression can provide input even after an apparent lysogenic decision, underscoring the importance of timing during decision-making (44, 45). Notably, almost all lambda research has been performed using the ΔparA strain, which contains a frameshift mutation in the stf gene, truncating the side tail fibers (46). Ur-λ is the actual WT strain with side tail fibers and has been shown to absorb faster to the E. coli cell surface (46, 47). It is still unknown whether having the side tail fibers would give Ur-λ more advantages in the DNA ejection process, either to facilitate or synchronize the ejection timing. Nevertheless, future studies monitoring the exposure of phages to cells, such as via microfluidic devices, combined with temporal tracking of desired phage biomolecules might be required to further assess the influence of timing in phage decision-making.

Biophysical properties of phages in vivo

More and more research focus on the physical properties of the bacterial cytoplasm (48–53), yet how the phage biomolecules behave in the cytoplasm remains poorly understood. As phage decision-making is affected by intracellular factors such as protein concentration and phage–phage interactions (38), studying the intracellular dynamics of those components could aid the understanding of lambda development. Some of the subcellular properties of E. coli biomolecules are known, but how do those conclusions relate to lambda? The extent to which lambda asserts itself versus reacting to the natural procession of the host may yield novel insights into decision-making.

Gene expression allows information to be passed from DNA to RNA and protein. The localization and cellular dynamics of the components along the path of gene expression may play important roles in determining the development of organisms. The bacterial cell was once thought as a bag of well-mixed enzymes, before the age of single-cell studies. It is now accepted that bacteria can have very well-organized protein localization (54). Homologs of the cytoskeletal proteins in eukaryotic cells are found in bacteria, and they regulate the spatial distribution of bacteria cells in sophisticated ways (55). Related investigations of cellular organization have spawned and been aided by techniques to label and track single RNA and protein molecules in vivo (56).

Both normal and anomalous diffusion have been reported for particles residing in the bacterial cytoplasm (49, 57), indicating complexity in the cytoplasm. Recently, a more systematic study has reported that the cytoplasm possesses glass-like properties and affects the motion of particles in a size-dependent manner, promoting heterogeneous motion in single cells (48). These characteristics are likely due to extreme crowding by macro-molecules (50, 51, 58). Those macromolecules, or crowders, can either be mobile or fixed in a network to result in the reduction of available solvent volume and the retardation of the motion of particles traveling within (52, 53, 59). This volume exclusion effect can cause microenvironments to form, where the local mRNA production rate exceeds the diffusion rate, causing a heterogeneous distribution of miRNA (60). In silico studies have also shown that spatial fluctuations of transcription factors can drastically increase noise in gene expression (61). Whether the physical properties of bacterial cytoplasm affect the diffusion of phage genomes and their relevant gene products remains to be investigated, and whether macromolecular crowding has any effect on the lysis–lysogeny decision-making of lambda awaits further study.

Recent development of fluorescence labeling techniques has allowed the tracking of lambda DNA, in conjunction with other desired molecules, in live E. coli cells (34, 62). One effort to track the lambda DNA in vivo utilizes the ParB/parS system (62). By inserting the parS sequence into lambda DNA, the phage locus can be tracked when ParB-FP fusion proteins are expressed in the host cell, as ParB polymerizes at parS sites (63–66). Using this method, lambda DNA was shown to reside within a small area close to its ejection site (62). Similar biophysical behaviors of lambda DNA were also observed using the
SeqA-FP labeling system (34). Shortly after infection, phage DNA was found to exhibit subdiffusive behavior, indicating restricted motion. Moreover, phage motion is not only heterogeneous in different cells, but also varies for different DNAs within the same cell and the same DNA at different time points in the infection course. It is possible that the dynamics of phage DNA can lead to different degrees of interaction between different infecting phages, therefore generating noise in infection outcomes.

Using the ParB/parS system, researchers observed that in lysogenic cells, the bacterial attB locus migrated toward the largely stationary phage DNA over time to possibly facilitate integration (62). A similar behavior was observed using the SeqA-FP system to label lambda DNA and a tetO/TetR system to label bacterial attB in lysogenic cells (37). Even in lytic cells, attB generally converges with lambda DNA-rich regions in an integrase-independent manner, leading to more co-localization of attB and lambda DNA (37). As lambda interacts with a variety of host factors, the impact of their organization on phage development can be further explored in single cells via fluorescence labeling.

The intracellular localization and biophysical behaviors of lambda RNAs are largely uncharacterized, but insights into this might be gained by examining the general behaviors of RNA in vivo. Using an MS2 coat protein–based system (67–70), the biophysical properties of mRNA molecules were characterized (70). In this study, the target RNA is engineered to have repeats of MS2-binding operator, which can be specifically bound by fluorescent MS2 coat fusions (70). Utilizing this method, mRNAs were shown to often exhibit localized motion and not leave a given area, possibly because they are tethered to the DNA template during transcription (70). Transcripts of some genes have also been reported to remain near their transcription sites and exhibit limited dispersion (71). Other mRNAs move within the cell and were found to localize to particular subcellular domains where their protein products function (72) or move to and remain in ribosome-rich areas (71, 73). Future research using techniques such as fluorescent labeling to study the distribution of phage RNAs might improve the understanding of decision-making at a systematic level. Alternatively, FISH is also a powerful tool to quantitatively probe transcription in single, fixed cells. Multiple transcripts may be labeled to study multiple processes in one cell to study the connections between different pathways.

To study proteins in live cells, fluorescent proteins are commonly fused to the desired targets. Regarding lambda decision-making proteins, there is concern that such fusions may interfere with their oligomerization or DNA-binding and looping functions, making quantitation difficult in single cells. To overcome this, techniques such as CoTrAC (co-translational activation by cleavage) can be used, where the fused fluorescent tag can be cleaved from target proteins after translation. In fact, research using this method to study the CI-Cro switch has revealed new cell-fate potentials for phage development (16). Alternatively, in fixed cells, immunofluorescence has been used to study proteins, and this method can be combined with RNA FISH, such that RNAs and proteins can be studied concurrently (74). The location and motion of DNA, RNA, and proteins of phage lambda in their native contexts are not well-characterized and represent a promising area of research. There are separated tools to target lambda biomolecules and corresponding portions of information, but no model that unifies lambda cell-fate selection. Linking together the elements of lambda decision-making will yield valuable details about how the pieces function as part of the whole process.

Perspectives

With the ability to investigate single cells, we now know how specific aspects of the lambda network impact its overall function, how phages can interact as subcellular individuals to decide cell fates, and how biological phenomena can emerge from previously overlooked behaviors of basic biomolecules. This story is still unfinished, but undoubtedly, its continuation will be supported as technology advances. Looking ahead, superresolution microscopy represents a new frontier for biology, where techniques to image specific DNA, RNA, and proteins have been developed for use in bacteria (75–78). The exquisite spatial resolution offered by such techniques should be vital for characterizing the “where” of lambda biomolecules to complement our knowledge on the “what,” “when,” and “how” aspects of decision-making.

Finally, it is important to recognize that lambda is one of myriad decision-making systems. Considering other phages, it is clear that many strategies have evolved for viral cell-fate selection, each an enticing target for high-resolution study. The temperate phage, P1, differs greatly from lambda. P1 lysogenizes as a plasmid, and its decision is reported to be independent of MOI (79–82). As a paradigm E. coli phage with a defined genetic network, P1 is perhaps ripe for high-resolution interrogation as a foil to lambda. Single-cell studies have been conducted for other phages as well, such as Salmonella typhi-murium phage, P22, exposing a novel carrier state, a behavior diverging from the classical lytic–lysogenic binary decision (83–85). This refinement of the established P22 decision-making model echoes the effect of high-resolution studies on lambda. Another example of unusual behavior is found in Bacillus subtilis phage, phi3T, which is reported to communicate using small molecules from cell to cell to influence its decision (86). Perhaps it might be possible to capture this fascinating process between single cells. It is worth mentioning that most bacterial systems lack the breadth of genetic tools enjoyed by E. coli. By employing labeling methods where the fluorophores, such as fluorescent dyes (87, 88) and particles (89–91), can be chemically modified to bind to phages, the burden of genetic engineering may be alleviated. Altogether, the results generated from high-resolution investigations inspire confidence in adopting such methods to resolve the many mysteries of phage biology.

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