Single-Cell Studies of Phage λ: Hidden Treasures Under Occam's Rug

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Abstract

Studies over more than half a century have resulted in what some consider a complete narrative for the life cycle of bacteriophage λ. However, this narrative is only complete within the limited resolution offered by the traditional genetic and biochemical approaches that were used to create it. A recent series of studies performed at the single-cell and single-phage levels has revealed a wealth of previously unknown features. By pointing to many open questions, these new studies highlight the limitations of our current understanding of λ, but they also initiate the process of forming a more detailed and quantitative narrative for the system.

INTRODUCTION: THE ILLUSION OF A COMPLETE NARRATIVE

Bacteriophage λ has played a historical role in the development of molecular biology (1, 2) and has served in establishing the basic paradigms for cellular processes such as DNA replication, homologous recombination, and transcriptional regulation (recently reviewed in 2). A great body of work, spanning more than half a century (3, 4), culminated in what many consider a complete narrative for the life cycle of the phage. This narrative begins with the docking of phage particles at the *Escherichia coli* cell surface and the injection of λ DNA into the cell. It proceeds with the decision between virulent (lytic) and dormant (lysogenic) pathways and, depending on the outcome of this decision, either viral reproduction and cell lysis or integration into the host genome and maintenance of the lysogenic state. Lysogenic cells are able to switch back to lysis (induction) if the cellular SOS response is activated (reviewed in 5). Because the existing narrative was supposedly complete, the implication was that it is about time to move on, leaving λ behind and proceeding to explore other systems [accordingly, the second edition to Ptashne's seminal book on λ had the title expanded to include "higher organisms" (6)].

However, the appearance of completeness is misleading. The canonical narrative is only complete within the level of description that is offered by the methods used to devise this narrative, mostly traditional genetics and biochemistry. The argument for completeness is thus circular. The corollary is that if the λ system were to be probed using newer methods, which allow for an increased level of detail, one should expect novel features to be revealed and new questions to arise. This is indeed what has happened over the past decade, when researchers went beyond bulk methods and began to probe the interaction between λ and its host in singulo (7), at the resolution of individual phages and cells, and coupled the experimental examination with quantitative and theoretical analysis. As I describe below, this line of investigation has led to new insights regarding multiple aspects of the λ life cycle (**Figure 1**): the way that the phage finds its target receptor on the cell surface (8, 9), the injection of phage DNA into the cell (10), the interaction between phage and bacterial genomes (11) and between individual phage genomes (12), the outcome of the postinfection decision (12, 13), the maintenance of lysogen stability (14), and the kinetics of lytic induction (15).[1]

We are reminded yet again of the important distinction between Occam's razor and Occam's rug (18). The former is a useful tool in physics, guiding us to choose the simplest explanation for any observed phenomenon. The latter, in contrast, is a warning for all practitioners of biology: A simple, elegant narrative typically implies that many details were left hidden under the rug. Single-cell studies of λ have begun to reveal some of the treasures that were left hidden under Occam's rug. These treasures are the focus of this review. Some older single-cell studies have already been reviewed (see 18), and I therefore focus on more recent ones.

ENABLING METHODS: SINGLE-CELL AND SINGLE-PHAGE REPORTERS FOR THE λ LIFE CYCLE

Over the past fifteen years, microscopy-based single-cell methods for quantifying cellular processes have gained popularity and have continuously improved in terms of accuracy and resolution (19, 20). As part of this trend, similar methods for probing the λ life cycle were also introduced. Single-cell studies of λ were first limited to measurements at the whole-cell level, but they gradually zoomed in to the subcellular level, down to individual phages and individual molecules within

[1]In addition to single-cell methods, genome-wide techniques have also been used successfully to reveal new players and regulatory relations in λ (16, 17). These works are outside the scope of this review.

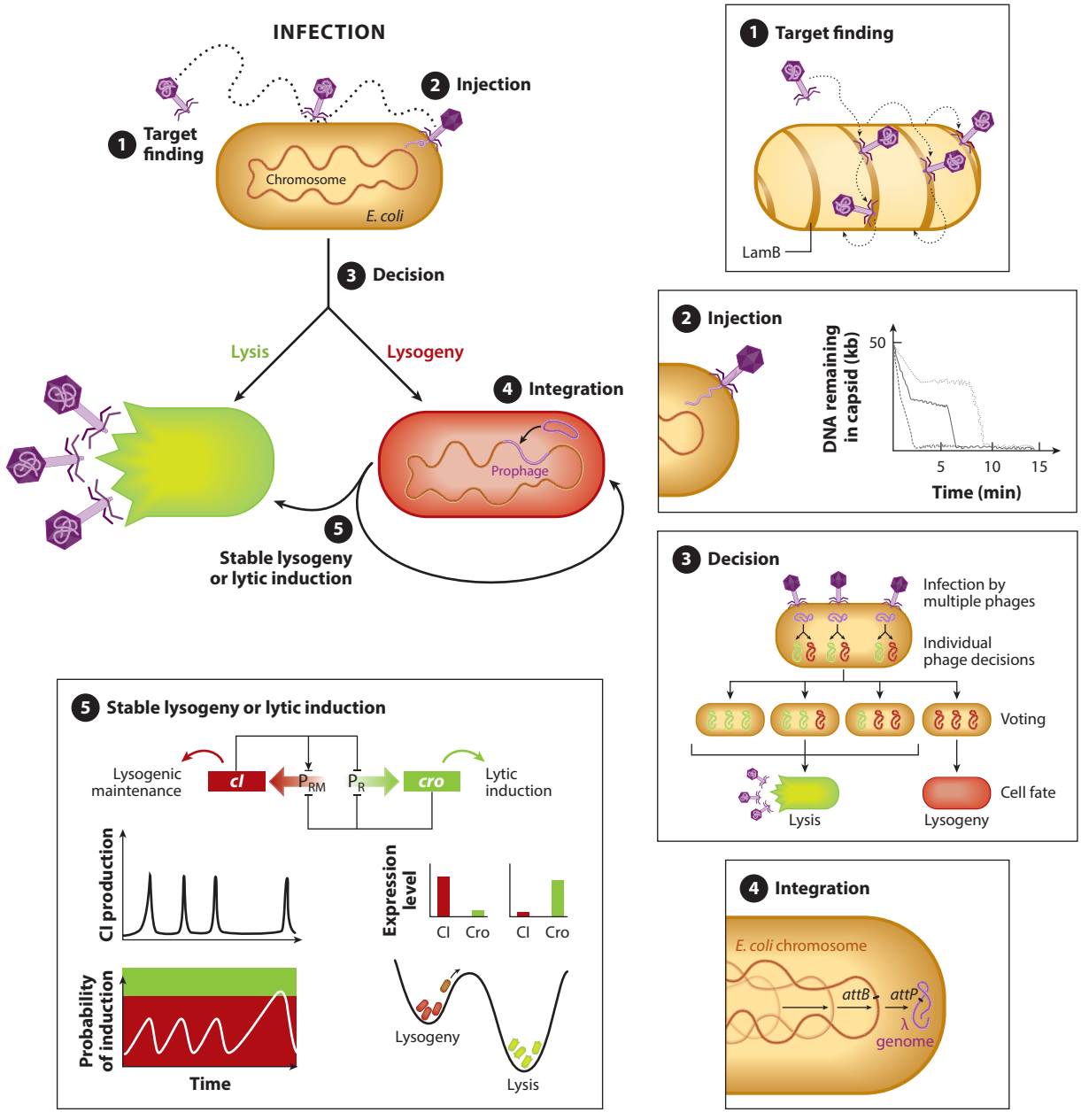


Figure 1

Novel features revealed by single-cell studies of the λ life cycle. (❶) Target finding. The infecting phage follows the helical pattern of LamB receptors on the cell surface, to end at the cell pole. (❷) Injection. DNA injection kinetics are slow, intermittent, and heterogeneous. (❸) Decision. During multiphage infection, the lysis/lysogeny decision first occurs at the single-phage level. Only if all phages choose lysogeny is that fate chosen for the cell; otherwise, lysis ensues. (❹) Integration. To allow lysogenic integration, the bacterial locus moves toward the stationary phage genome. (❺) Stable lysogeny or lytic induction. (*Bottom left*) The stability of the lysogenic state is determined by the frequency of *cI* transcription bursts. (*Bottom right*) The lysis/lysogeny system is bistable.

Table 1 Enabling methods for studying λ infection at the single-cell level

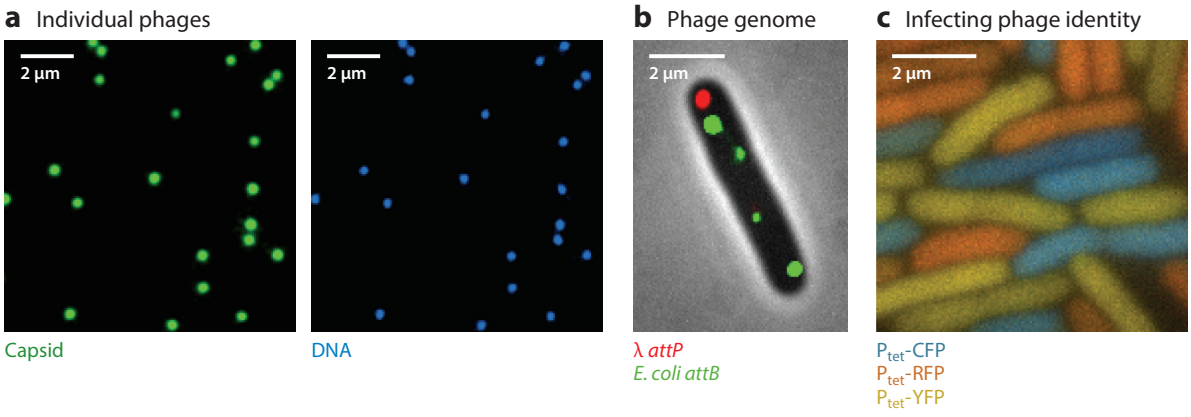
Labeled entity	Labeling principle	Questions addressed	Image	Video
Infected cell	Nonfluorescence light microscopy	Cell fate; timing of cell lysis (21, 22)	**Figure 2*d*,*e***	**Video 1**
Gene expression	Promoter fusions to fluorescent proteins	Activity of multiple λ promoters during infection, during induction, and in a lysogen (12, 15, 21, 28); phage identity during multiphage infection (Golding lab, unpublished results)	**Figure 2*c*,*d***	**Video 2**
	MS2-GFP labeling of individual mRNA	P_{RM} kinetics in a lysogen (31); P_R activity during induction (Golding lab, unpublished results)	**Figure 2*g***	**Video 3**
	Single-molecule imaging of fluorescent proteins	P_{RM} kinetics in a lysogen (35)	—	**Video 4**
	Single-molecule fluorescence in situ hybridization (smFISH)	Activity of multiple λ promoters during infection, during induction, and in a lysogen (14, 34; Golding lab, unpublished results)	**Figure 2*f***	—
Phage capsid	Quantum dots	Position of phage docking site on the cell surface (8)	—	—
	gpD-YFP fusion	Phage movement on the cell surface (9); postinfection decision (12, 37)	**Figure 2*a*,*d***	**Video 5**
Phage genome	DNA dye	Injection kinetics (10)	**Figure 2*a***	**Video 6**
	DNA-binding protein fused to a fluorescent protein	Spatiotemporal dynamics of λ genome in the cell (11, 38); DNA looping (40)	**Figure 2*b***	**Videos 7** and **8**

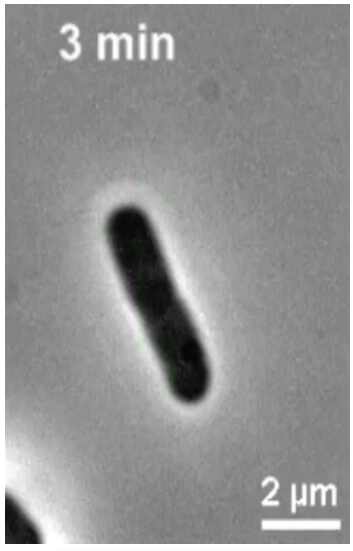
Figure 2

Bacteriophage λ infection at the single-cell level: sample images (see **Table 1**). (*a*) Individual phage particles. (*Left*) Each phage capsid (*green*) contains a mixture of wild-type gpD protein and a gpD-YFP fusion. The phages are detected as diffraction-limited spots under epifluorescent illumination. (*Right*) DAPI staining of phage DNA (*blue*) colocalizes with the YFP signal. See Reference 12. (*b*) Phage (*red*) and bacterial (*green*) genome loci inside the infected *Escherichia coli* cell (see also **Video 7**). The phage genome is detected by inserting a *parS* sequence near the *attP* site. The sequence then becomes bound by multiple mCherry-ParB proteins. In the same cell, the bacterial *attB* site is detected using an orthogonal *parS*/ParB system and a different fluorescent protein. Note that the infected cell has four copies of the *attB* locus. See Reference 11. (*c*) The identities of individual infecting phages can be distinguished by the expression of different fluorescent proteins from the phage genome. Here, phages encoding CFP, RFP, and YFP were created and used for infection. (*d*) Tracking the postinfection decision. Frames from a time-lapse video depicting multiple infection events (see also **Video 2**). Phage capsids are labeled using gpD-YFP. At $t = 0$ min (*left*), two cells are each infected by a single phage (*green spots*), and one cell is infected by three phages. At $t = 80$ min (*middle*), the two cells infected by single phages have gone into the lytic pathway, as indicated by the intracellular production of new phage capsids (*green*). The cell infected by three phages has gone into the lysogenic pathway, as indicated by the activity of a P_{RE}-mCherry reporter plasmid (*red*). At $t = 120$ min (*right*), the lytic pathway has resulted in cell lysis, whereas the lysogenic cell has divided. See Reference 12. (*e*) Cell lysis following infection, as seen under phase-contrast illumination (see also **Video 1**). The two images were taken 80 and 90 min after infection, respectively. (*f*) *cI* mRNA in lysogenic cells. Single-molecule fluorescence in situ hybridization (smFISH) was used to label individual *cI* mRNAs in chemically fixed cells. See Reference 33. (*g*) P_R activity in live cells (see also **Video 3**). The P_R promoter was fused to 24 binding sites for the MS2 coat protein. The transcribed RNA becomes decorated with multiple MS2-GFP proteins (31). The genome locus where the reporter resides contains an array of 140 *tetO* sites and is detected through the binding of TetR-mCherry (83). The cells do not express CI, resulting in strong P_R activity. Panels *b*, *c*, *e*, *f*, and *g* courtesy of Louis McLane, Leonardo Sepúlveda, Samuel Skinner, and Jing Zhang; Golding lab (unpublished). Panels *a* and *d* adapted from Reference 12, copyright 2010, with permission from Elsevier.

the infected cell. I begin this review by surveying key single-cell methods that have been used successfully for studying λ. These are also summarized in **Table 1**, **Figure 2**, and **Videos 1–8**. The key results that came out of these studies are discussed in the following section.

At the whole-cell level, simple nonfluorescent light microscopy can be used to follow the fate of individual cells after infection. Cell lysis can be readily observed under the microscope





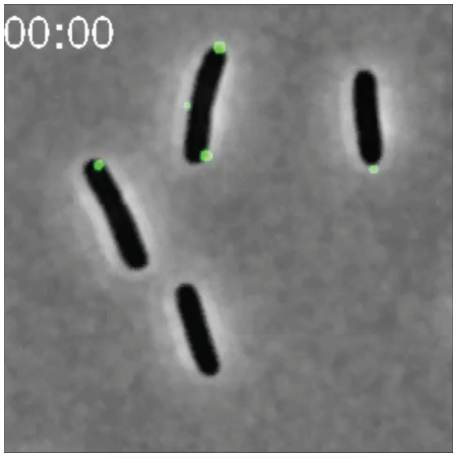
Video 1

Cell lysis following λ infection, as seen under phase-contrast illumination (see also **Figure 2*e***). The cell is infected at $t = 0$ and lyses at $t \approx 80$ min. Video courtesy of Louis McLane and Samuel Skinner; Golding lab (unpublished).

(**Figure 2*e***; **Video 1**). This type of single-cell characterization has been in use for many years (1). More recently, several studies have used this approach to investigate the origins of cell-to-cell variability in lysis time (21, 22). Phages that carry a selection marker, such as an antibiotic resistance cassette (23), can be used to identify lysogenic cells by subjecting the infected cells to selection such that only lysogens are able to grow (L. McLane, Golding lab, unpublished results).

Although regular light microscopy allows us to observe individual cells, the key enabling technology for making measurements on these cells is fluorescence microscopy. In *E. coli*, promoter fusions to fluorescent proteins have been used with great success to describe the kinetics of expression for many genes, at both the population (24) and single-cell (25) levels. The same approach was used for following some of the key λ promoters both in bulk (26, 27) and in singulo (28). Amir et al. (21) and Zeng et al. (12) examined the activity of several promoters along the paths to lysis and lysogeny (**Figure 2*d***; **Video 2**). Bednarz et al. (15) demonstrated the bistability of the lysogeny maintenance gene circuit and measured the kinetics of lytic induction. Using a similar approach, my laboratory recently used phages encoding different fluorescent proteins to distinguish individual phages during a multiphage infection (**Figure 2*c***).

The ability to probe viral gene expression improved significantly with the introduction of single-molecule measurement techniques—again following their successful application for bacterial genes (20, 29). mRNA can be labeled and quantified in individual live cells by fusing the promoter of interest to an array of binding sites for the MS2 coat protein, and expressing an MS2-GFP fusion in the same cells (30). This scheme was used to probe the kinetics of mRNA production from the lysogeny maintenance promoter P_{RM} (31) and more recently the lytic promoter

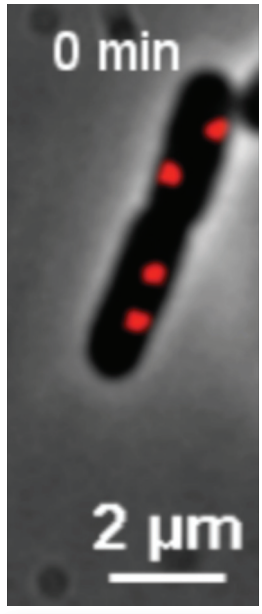


Video 2

Tracking the postinfection decision (see also **Figure 2*d***). Phage capsids were labeled using gpD-YFP. At $t = 0$, two cells are each infected by a single phage (*green*), and one cell is infected by three phages. The two cells infected by single phages go into the lytic pathway, as indicated by the intracellular production of new phage capsids (*green*). The cell infected by three phages goes into the lysogenic pathway, as indicated by the activity of a P_{RE}-mCherry reporter (*red*). Eventually, the lytic pathway results in cell lysis, whereas the lysogenic cell continues to grow and divide. Video reproduced from Reference 12, copyright 2010, with permission from Elsevier.

P_R (**Figure 2*g***; **Video 3**). In chemically fixed cells, single-molecule fluorescence in situ hybridization (smFISH) (32, 33) can be used to count endogenous transcripts at single-molecule resolution (**Figure 2*f***), and the copy-number statistics can in turn be used to estimate the stochastic kinetics of promoter activity (33, 34). Zong et al. (14) used this approach to characterize P_{RM} and P_R activity and relate it to lysogen stability. At the protein level, Hensel et al. (35) used single-molecule imaging to directly follow the production of CI (λ repressor), the transcription factor responsible for maintaining the lysogenic state (**Video 4**).

Some of the most exciting new discoveries regarding the λ life cycle have come from the ability to probe it at the resolution of individual phages. To fluorescently label the viral capsid, Edgar et al. (8) conjugated quantum dots to λ phages (as well as to other bacteriophages). They then examined the spatial position of infecting phages on the cell surface. Zeng et al. (12) used an alternative approach to label λ capsids. Building on earlier efforts (36), they created hybrid phages, in which the phage capsid contains a mixture of the wild-type head-stabilization protein gpD and a fusion protein of gpD to YFP. In the later version of this method (37, 38), gpD-YFP is provided from a plasmid during lysogen induction. The lysogen itself can be of any genotype; thus, the method allows one to fluorescently label any λ mutant without the need to manipulate the phage genome. Individual phages are detected as diffraction-limited spots under epifluorescence microscopy (**Figure 2*a***). Zeng et al. (12) used these phages to characterize how the postinfection decision between lysis and lysogeny depends on the number of coinfecting phages (multiplicity of infection; MOI) (**Video 2**). Rothenberg et al. (9) used similarly labeled phages to examine how infecting phages find their target receptor on the *E. coli* cell surface (**Video 5**).



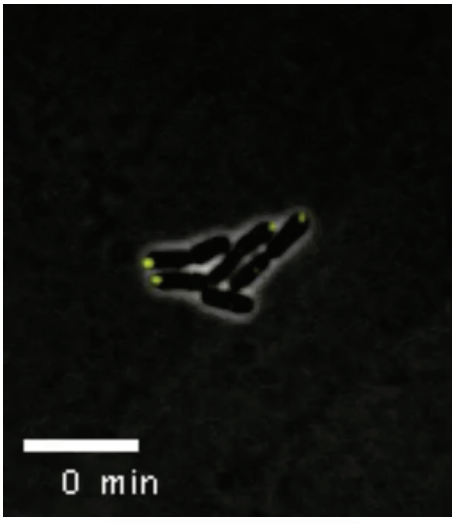
Video CLICK TO VIEW

Video 3

P_R activity during lysogen induction (see also **Figure 2*g***). In the reporter strain, mRNA from the P_R promoter is labeled using MS2-GFP (*green*), whereas the genome locus where the reporter resides is labeled using TetR-mCherry (*red*). The cells are λ lysogens. At $t = 20$ min, mitomycin C is added, leading to transient derepression of P_R and induction of the dormant prophage. The prophage used is lysis deficient; hence, the cells do not lyse at the end of the induction process. Video courtesy of Jing Zhang, Louis McLane, and Samuel Skinner; Golding lab (unpublished).

During the intracellular phase of the life cycle, the phage is first present only in the form of its genome. A number of approaches have been used to label individual viral genomes and examine DNA entry into the cell and its subsequent intracellular dynamics. Fluorescent DNA dye can be used to label the λ DNA inside the capsid (**Figure 2*a***) (12). Van Valen et al. (10) used the intensity of the fluorescent signal to quantify the kinetics of DNA injection into the cell (**Video 6**). Edgar et al. (8), following the approach of Lau et al. (39), engineered an array of *lacO* sites into the λ genome. Expressing LacI-YFP in the infected cell allowed them to detect the spatial position of the injected phage genome inside the cell. Similarly, Hensel et al. (40) engineered short arrays of *tetO* and *lacO* sites next to the proximal and distal operators of the P_{RM} promoter. They then expressed the fluorescently labeled cognate proteins and used two-color imaging to probe DNA looping between the two operator sites. Pursuing an analogous approach, Tal et al. (11) (following 41) used *parS* sites and ParB-mCherry to follow the spatiotemporal dynamics of phage genomes en route to integration into the bacterial chromosome during the lysogenic pathway (**Figure 2*b***; **Video 7**). Shao et al. (38) used a SeqA-CFP fusion, which binds to methylated DNA, to label a subset of the infecting phage genomes and follow their movement in the cell (**Video 8**).

One of the most promising experimental directions for interrogating λ infection at the single-cell level is the combination of multiple observables within the same cell, something that has been done on only a limited scale so far. For example, in the study by Zeng et al. (12), the



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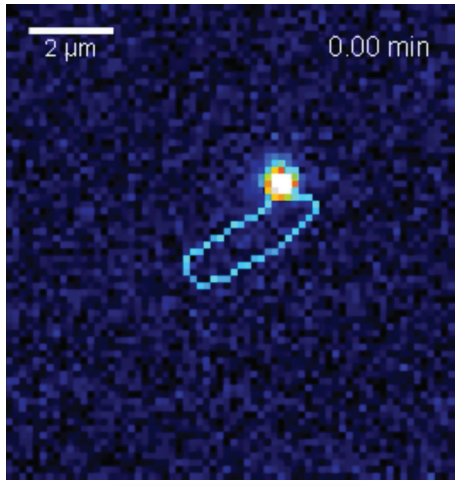
Video 4

Single-molecule imaging of CI production (*yellow*) during stable lysogeny. For more information see Reference 35. Video reproduced from Reference 35, copyright 2012, with permission from Macmillan Publishers Ltd.

Video CLICK TO VIEW

Video 5

A single gpD-YFP-labeled λ phage (*green*) diffusing near and on the *Escherichia coli* cell (*red*). Two examples are shown sequentially. For more information see Reference 9. Video courtesy of Eli Rothenberg, Samuel Skinner, and Ido Golding (unpublished).



Video 6

DNA injection into the cell. The λ genome, initially inside the capsid, was labeled using a SYTOX Orange DNA dye. For more information see Reference 10. Video reproduced from Reference 10, copyright 2012, with permission from Elsevier.

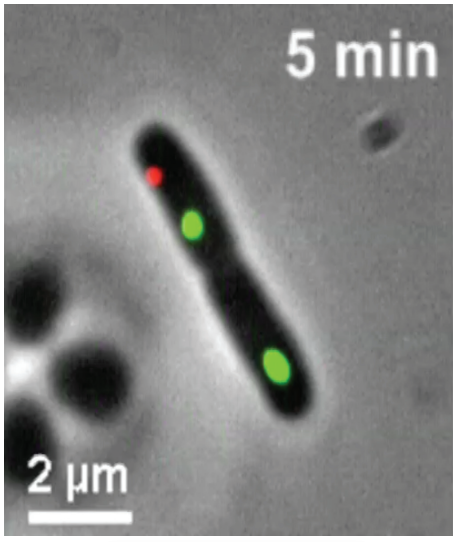
choice between lytic and lysogenic pathways in a given cell was distinguished by the expression of gpD-YFP or of mCherry, from the $P_{R'}$ or P_{RE} promoters, respectively (**Figure 2*d***; **Video 2**). More recently, Shao et al. (38) combined YFP labeling of the phage capsid with SeqA-CFP detection of the phage genome inside the cell (**Video 8**). By labeling DNA loci on both the λ and *E. coli* genomes, Tal et al. (11) were able to examine how the two find each other during prophage integration (**Figure 2*b***; **Video 7**). Finally, the dynamics of the genetic circuit composed of *cI* and its antagonist *cro* was examined using two-color smFISH (14) as well as two-color fluorescent promoter fusions (15).

The innovative methods highlighted above have been used with great success to investigate different aspects of the λ life cycle. This examination at the level of individual cells and phages has repeatedly led to surprising discoveries and novel insights. These are discussed next.

KEY DISCOVERIES

Viral Target Search

In the first step of infection, the phage docks at a target receptor on the surface of the host cell. For λ, this receptor is the LamB protein, used by *E. coli* for maltose uptake (42, 43). The spatiotemporal dynamics by which a phage arrives at its target receptor on the cell surface has served as a paradigm for diffusion-driven target finding in biology (44, 45) and, for λ, had been examined meticulously using bulk methods (46). These studies indicated that target finding occurs in two distinct kinetic steps. The first step was interpreted as corresponding to the arrival of the phage, by three-dimensional diffusion, from bulk liquid to the cell surface. The second step was assumed to consist of random two-dimensional motion of the phage on the cell surface, until it encounters one of the thousands of LamB proteins on the surface and proceeds to inject its DNA.

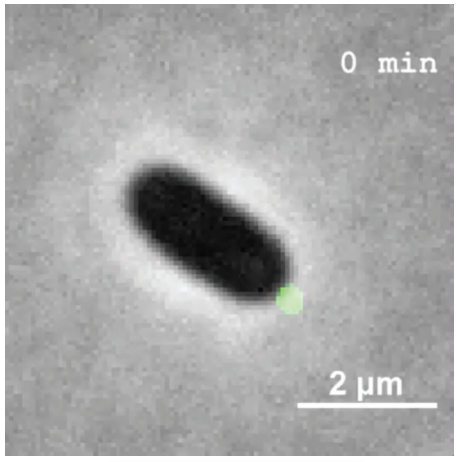


Video 7

Spatiotemporal dynamics of phage (*red*) and bacterial (*green*) genome loci inside the infected *Escherichia coli* cell during the lysogenic pathway (see **Figure 2*b***). The phage genome is labeled using mCherry-ParB. In the same cell, the bacterial *attB* site is detected using an orthogonal ParB system and GFP. For more information see Reference 11. Video courtesy of Louis McLane and Samuel Skinner; Golding lab (unpublished).

However, once phage-host interactions were examined at the single-cell level, the picture of a simple diffusion-based random search was challenged. Two separate studies found that the positions of both the LamB receptors and the docking phages are far from being uniformly distributed on the *E. coli* surface. Using fluorescently labeled phage tails as a proxy for the presence of LamB proteins to which they bind, Gibbs et al. (47) found that these receptors form approximately helical patterns on the cell surface, similar to those exhibited by other bacterial proteins (48), and that these patterns change with time. In terms of the infecting phages themselves, Edgar et al. (8) found that λ (as well as other phages) shows a strong preference for docking at the cell pole, as well as the mid-cell area (**Figure 2*d***; **Videos 2** and **8**). These observations reopened the question of how the landing phage finds its docking site on the cell surface.

To directly address that question, Rothenberg et al. (9) followed individual λ phages in real time, from when they arrived at the *E. coli* cell surface until they docked. The authors found that the search pattern was neither uniform nor isotropic, as would be expected for random diffusion. Instead, the phage trajectories followed helical patterns (**Figure 1**, item ❶; **Video 5**). Simultaneous labeling of the phage and LamB revealed that phage trajectories followed the underlying pattern of LamB on the cell surface. In other words, LamB proteins are not merely the final destination of λ but somehow guide the search process itself [something that was already hinted by earlier bulk results (46)]. In mutant *E. coli* missing LamB, the arriving phages did not search the surface at all but instead fell off it immediately. In the presence of LamB, the surface exploration lasted for ∼1 s and resulted, in ∼50% of the cases, in the phage finding a final docking site. This site showed a preference for the cell pole, as previously found by Edgar et al. (8). In the other ∼50% of infection events, the phage fell off the surface and went back into the bulk liquid.



Video 8

Spatiotemporal dynamics of the phage genome inside the cell during the lytic pathway. At $t = 0$ min, the cell is infected by a gpD-YFP-labeled phage (*green*). At $t = 5$ min, a SeqA-ECFP focus corresponding to the injected phage DNA appears. Later, this focus is converted to two foci, corresponding to two hemimethylated phage DNAs. The cell eventually lyses (not shown). Video reproduced from Reference 38, copyright 2015, with permission from Elsevier.

The findings of Rothenberg et al. (9), though they provided rich new phenomenology, leave multiple open questions regarding the process of target finding by the infecting phage. To begin with, how are the helical patterns of LamB protein formed? As indicated above, similar patterns are exhibited by other bacterial proteins, notably the actin-like protein MreB (48), but their origin is still unresolved. Next, how does λ hop from one receptor to the next, and how does this movement result in the phage eventually arriving at the cell pole? On the longer timescale, how has this intricate search algorithm evolved, and how does it reflect the ongoing arms race between the phage and its host (49)?

DNA Injection into the Cell

After docking, the phage next injects its DNA into the infected cell, leading to the expression of viral genes. The kinetics of DNA injection remained unclear until recently. Mackay & Bode (50) suggested that the process might take from a few minutes to almost an hour, depending on temperature. Their study also suggested significant variability in the kinetics of individual infection events. Unfortunately, the population-based methods used in that study did not enable the authors to delineate phage adsorption, the triggering of DNA injection, and the kinetics of DNA entry into the cell, instead providing a coarse-grained measurement of all these processes combined. Their observation of a possibly very slow injection process was also contradicted by later in vitro studies at single-molecule resolution, which found that the complete λ DNA is ejected in as little as ∼10 s (51).

To resolve this conflict and characterize the kinetics of DNA injection at the resolution of individual phages and cells, Van Valen et al. (10) labeled the λ genome using a fluorescent DNA dye and tracked the decrease in phage DNA signal inside the capsid, together with the corresponding increase of signal inside the cell (**Figure 1**, item ❷; **Video 6**). By tracking many individual injection

events, the authors found that injection was indeed almost two orders of magnitude slower than measured in vitro, taking on average about 5 min to complete. As also foretold by the older study (50), individual injection events varied significantly in their duration. Moreover, many injection trajectories included pauses that lasted a few minutes. In addition to unveiling the kinetics of viral injection, Van Valen et al.'s study provided hints into the underlying biophysics, by showing that the instantaneous injection velocity depended on the amount of DNA already in the cell. Nevertheless, the mechanism of phage injection remains to be elucidated, as does the source of cell-to-cell heterogeneity in injection kinetics.

The Lysis/Lysogeny Decision and the Intracellular Dynamics of Viral Genomes

Once the λ genome has entered the cell, phage-encoded genes are expressed, and the viral developmental program is executed. The first step in this program is the choice between two possible pathways: the lytic pathway, consisting of viral reproduction and cell death (lysis), and the lysogenic pathway, resulting in integration of the λ genome into the bacterial chromosome to become a dormant prophage (5). The lysis/lysogeny decision is perhaps the most famous aspect of the λ life cycle and is considered a simple paradigm for cell-fate choice driven by a genetic circuit (5, 18). As in other parts of the life cycle, the key genetic players and many—but probably not all—of the interactions among them have been identified over the years. This knowledge infrastructure has allowed researchers to form mathematical models of the decision process, at different levels of detail (52–56). These models were capable of reproducing some of the phenomenology of the system—in particular, the observation that the genetic circuit commits to lysogeny if the number of coinfecting phages exceeds a given threshold (57).

However, this apparent success is misleading. In fact, the lysis/lysogeny decision is quite complex in terms of both the underlying circuitry and the resulting dependence on infection parameters. In addition to the control of transcription initiation, critical regulation occurs at the levels of transcription termination and protein degradation, with important contributions from multiple host factors interacting with the phage-encoded players (2, 58). Some of these regulatory interactions remain unresolved. As a demonstration of our still limited understanding, the decision outcome exhibits strong dependence on the physiological state of the infected cell [e.g., growth rate (13, 57)] but this feature has not yet been quantitatively characterized or reproduced by theoretical models. Thus, we are still far from having predictive power even for this supposedly simple circuit.

As in other aspects of the λ life cycle, recent single-cell studies have revealed surprising features of the postinfection decision and have pointed to unresolved questions. One such question is the role played by biochemical stochasticity ("noise") in the choice between lysis and lysogeny. The λ decision has been put forward as a founding paradigm for noisy cell-fate decisions: a scenario in which random fluctuations in the expression of fate-determining genes render cell-fate choices unpredictable ("noisy"), rather than deterministic, at the level of the individual cell (52). This idea became widely accepted in higher systems as well, for example, in the contexts of mammalian viruses (59) and of animal development (60). Ironically, the idea that the lysis/lysogeny decision is noisy was not based on any single-cell measurements but rather was inspired by theoretical arguments (52). In fact, once the decision process was examined at a finer resolution, the relative role of stochasticity began to erode, and additional deterministic factors driving the decision were revealed.

A first hint that the λ decision is not as noisy as suggested came from the work of St-Pierre & Endy (13). By fractioning cells into different sizes, the authors showed that, during infection by a single phage, the infection outcome is strongly correlated with cell size. Next, Zeng et al. (12) tracked λ infection at the resolution of individual phages and cells (**Figure 1**, item ❸; **Figure 2*d***;

Video 2). Analyzing the dependence of the infection outcome on the number of simultaneously infecting phages (MOI) and cell size, the authors concluded that the lysis/lysogeny decision is first made at the level of each individual phage, based on the total viral concentration in the cell (given by the ratio of MOI to cell volume). The single-phage decision is still noisy, but considerably less so than what is observed at the cellular level. The decisions by all coinfecting phages are integrated in a deterministic manner, such that only if all of them choose lysogeny is that path taken; otherwise, the lytic pathway is pursued, resulting in cell death. Another recent study (61) found further support for the picture of individual—and possibly conflicting—decisions by coinfecting phages.

The idea of phage individuality within the infected cell requires us to rethink how the genetic circuitry of the phage drives the lysis/lysogeny decision. In the traditional picture for this process, the gene products (mRNA and protein) from all phage genomes present in the same cell are assumed to rapidly mix via diffusion. Consequently, the distinction between individual genomes is erased. The fact that genome identity is actually maintained may reflect the significance of *cis* events occurring at each individual genome; for example, the antitermination of transcription from the late promoter $P_{R'}$, which results in the expression of lytic genes (58, 62).

Another possibility is that the ability to maintain genome individuality reflects hitherto unknown spatial effects, which perhaps prevent the mixing of gene products originating from different genomes. This type of effect has been proposed to allow viral individuality in higher systems (63). In λ, as discussed above, phages exhibit a nonuniform spatial distribution of docking sites, with a preference for the *E. coli* cell pole. Zeng et al. (12) found that when phages dock away from these preferred sites, the infection is more likely to fail (i.e., result in neither lysogeny nor lysis). Tal et al. (11) recently examined intracellularly how the λ genome (specifically, the *attP* site) finds its unique integration locus (*attB*) in the *E. coli* chromosome, en route to becoming a dormant prophage (**Figure 1**, item ❹; **Figure 2*b***; Video 7). The canonical picture for this process was of a diffusion-based random search, presumably involving a mixture of three-dimensional diffusion of the viral genome in the cytoplasm and one-dimensional sliding of DNA molecules on one another. Such behavior has been observed, for example, in the case of transcription factors searching for their cognate binding site (64, 65). This, however, was not what Tal et al. found. Following entry into the cell, the λ genome remains almost stationary. The bacterial locus, instead, is the one that moves, as part of the normal replicative cycle of the chromosome. This movement, in time, brings the bacterial and phage attachment loci in close proximity, allowing integration to proceed.

As part of their investigation, Tal et al. (11) found that moving the integration locus to a different position in the bacterial chromosome resulted in a diminished lysogenization frequency. This was a first indication that the unique search mechanism they uncovered was relevant to the outcome of the lysis/lysogeny decision, rather than merely affecting the process of integration after lysogeny has been chosen. One is then led to contemplate additional ways in which the phage and host genomes' spatiotemporal dynamics may affect the choice of cell fate. First, does the search set a time window in which the lysis/lysogeny decision is reversible? Svenningsen & Semsey (61) have shown that the decision can be reversed for up to ∼1 h following infection, a duration that is comparable to the search time found by Tal et al. (11), but whether the two are related remains to be examined. Second, does the dependence on bacterial chromosome movement create a coupling of the decision to the cell cycle of the infected cell? Multiple studies over the years have pointed to intriguing interactions between λ and the *E. coli* cell cycle (66, 67), but these interactions have not yet been examined at the resolution of individual phages and cells.

Currently, the spatiotemporal dynamics of the λ genome inside the infected cell, and the implications for the developmental program, remain unclear. Temporally, we do not know how much genome replication occurs prior to the lysis/lysogeny decision, nor do we know what effect this early replication has on the infection outcome. Early replication has been demonstrated in

older studies (68) and is possibly reflected in the common occurrence of multiple tandem prophage copies in a lysogenic cell (69, 70). Svenningsen & Semsey (61) recently suggested that the presence of multiple λ genomes at the time of the lysis/lysogeny decision renders the choice reversible until the fates of all individual genomes have been resolved. Spatially, we know very little about the intracellular organization of replicating phage genomes, as well as of the subsequent phage particles. Studies in other phages have pointed to intriguing spatial patterns (71), but this subject has only begun to be tackled in λ (8, 38).

Lysogenic Maintenance and Lytic Induction

Just as the initial lysis/lysogeny decision is considered a paradigm for cell-fate determination, maintenance of the lysogenic state serves as a prototype for long-term epigenetic memory of cellular state (18, 58, 72). This memory is propagated over many cell generations via the activity of CI, which represses all lytic functions while autoregulating its own expression (5, 73, 74) (**Figure 1**, item ❺). The lysogenic state combines two seemingly conflicting phenotypes. On one hand, it is extremely stable: Under optimal conditions, spontaneous switching to lysis occurs with a probability $<10^{-5}$ per cell generation (23). On the other hand, lysogenic cells will efficiently switch to lysis if CI is depleted or inactivated, a process called lytic induction (5, 58). These features of the λ circuit—autoregulation by the fate-determining gene, resulting in long-term stability combined with efficient switchability—are present in higher systems as well (75, 76). The obvious advantages offered by λ are its relative simplicity and experimental tractability, making it suitable for attempting to form a quantitative understanding of how cell fate is maintained and how it is altered. This understanding should be reflected in the ability to predict, based on the parameters of the genetic circuit, the system-level phenotypes: how stable the lysogenic state is, and how it responds to perturbations in CI.

To predict the stability of the lysogenic state, one needs to characterize the stochastic kinetics of CI production from the P_{RM} promoter. Even in the absence of any external perturbation, there would still be rare random events in which CI levels drop sufficiently such that the P_R promoter is derepressed, leading to production of the antirepressor Cro and initiation of the lytic pathway (14, 23, 77). To estimate how often such events will occur, Zong et al. (14) measured the number of *cI* mRNA molecules in individual lysogenic cells (**Figure 2*f***) and used the copy-number distribution to estimate the parameters of stochastic P_{RM} activity [other studies measured these kinetics directly in live cells, using reporter fusions to detect individual mRNAs (31) or proteins (35) (**Video 4**)]. Zong et al. also measured lysogen stability by assaying the appearance of phage particles in a growing population of lysogens. By repeating these measurements for both wild-type and mutant phages, the authors found a simple exponential relation between the spontaneous induction rate (the inverse of stability) and the frequency of *cI* transcription bursts. This finding was exactly as predicted by the simple picture in which random loss of CI, due to unusually prolonged periods of P_{RM} inactivity, results in lytic induction (**Figure 1**, item ❺).

Beyond this single calculation, a harder challenge is to predict the response of the system to any specific perturbation. In that regard, the lysis/lysogeny system has often been referred to as bistable (15, 78, 79). This term means that each of the two possible states, lysogeny and lysis (or more accurately, the onset of lysis), is locally stable: Small perturbations will not lead to a change in cell fate. Recently, Bednarz et al. (15) used a two-color reporter for P_{RM} and P_R activity to directly test the bistability hypothesis at the single-cell level. The authors found that the lysis/lysogeny system exhibits the hallmark phenotype of bistability, which is hysteresis: The state of each cell depends on its long-term (multigenerational) history, rather than simply on the current conditions. Thus, both lysogeny and the onset of lysis are indeed stable states of the system (**Figure 1**, item ❺).

Next, Bednarz et al. (15) used the reporter to follow the gene expression trajectory of the system during a forced switch from lysogeny to lysis. Specifically, they measured the fraction of induced cells as a function of time following heat inactivation of the CI protein. The observed kinetics were biphasic, characterized by a delay period followed by exponential switching. A stochastic mathematical model of the circuit was able to reproduce these features, lending credence to the theoretical picture of lysogenic maintenance.

Although they provide useful insight, Bednarz et al.'s (15) measurements, which involved taking snapshots of cell populations, still fall short of the type of experiments that would truly allow us to challenge the existing theoretical picture of the lysis/lysogeny system. Such experiments should simultaneously measure P_{RM} and P_R activity in individual cells, in real time, during the course of lytic induction (**Video 3**). The kinetic data will reveal whether induction, as suggested by the bistability picture, consists of the system first "climbing up" the barrier that is formed by the *cI*/*cro* circuit, then "sliding" into the neighboring attractor of lytic development (**Figure 1**, item ❺). It is quite possible that, as in other cases discussed in this review, achieving the desired experimental resolution will not validate the prevailing theoretical picture but instead will expose its inadequacy.

In fact, a number of open questions highlight gaps in our picture of the induction process. For example, does the system exhibit a mixed state, in which both *cI* and *cro* are expressed simultaneously? Such a state is not part of the canonical picture but was suggested by single-cell mRNA measurements (14), as well as by theoretical and experimental studies of a similar synthetic circuit (80). Another question concerns the way induction is triggered by *E. coli*'s SOS response. During that response, DNA damage leads to RecA activation, in turn triggering CI degradation (81). RecA activation involves complex spatial dynamics (82) as well as temporal oscillations (25), either of which may underlie—in ways yet to be elucidated—the dramatic cell cycle dependence of the induction efficiency, reported previously (67).

DISCUSSION: IN SEARCH OF A QUANTITATIVE NARRATIVE

The recent wave of single-cell studies discussed in this review is reminiscent of (and in some cases inspired by) the original work of the so-called phage group, which took place in the mid-twentieth century (1). Just like that first wave, this latest one is driven by the physics-inspired idea that the relative simplicity of the phage makes it amenable to precise experimental interrogation, which will ultimately lead to the development of a quantitative narrative for its life cycle. This endeavor will, in turn, lead the way to similar narratives for more complex organisms.

Standing on the shoulders of past λ giants, single-cell studies have revealed a wealth of hitherto unknown features regarding different aspects of the phage life cycle. In doing so, these studies have shown us how far we still are from forming a full quantitative narrative for the behavior of the system. Why is that? Physics teaches us that the only way to create a full narrative for a complex system is by elucidating a finite set of simple principles that underlie its function. Accordingly, many of the studies discussed here were motivated by the desire to identify such simple principles, or validate previously hypothesized ones. But instead, the experimental interrogations repeatedly revealed additional layers of complexity, pushing further away our hopes of finding simplicity.

One example of a case in which the attempts to validate a simple biophysical picture led instead to the discovery of more complex behavior is that of spatial target finding. Above, I discuss two cases: an infecting phage finding the LamB receptor on the cell surface, and the phage genome finding its integration site in the bacterial chromosome. In both cases, there existed a well-defined theoretical picture for how the search should take place. It invoked random diffusion, possibly enhanced by an effective switching between spatial dimensions as a means of minimizing the

search time (45) (from three dimensions to two dimensions for finding the surface receptor, and from three dimensions to one dimension for finding the integration locus). But as we have seen, what was found experimentally was very different from the expectations. Phages cover the *E. coli* surface in a nonuniform, anisotropic manner, and their trajectory follows the underlying spatial pattern of LamB proteins (9) (**Figure 1**, item ❶). And the λ genome waits statically for its *E. coli* counterpart while the latter undergoes directed, deterministic motion toward the cell pole (11) (**Figure 1**, item ❹). Thus, instead of both cases falling into a preexisting simple paradigm for biological target finding, each of them instead appears to present a new mechanism, one that requires additional experimental and theoretical interrogation.

Another example in which single-cell studies cast doubt on the prevailing paradigm rather than validate it concerns the role of noise in driving cell-fate decisions. Arkin et al. (52) argued that biochemical stochasticity renders the λ postinfection decision inherently unpredictable at the single-cell level. The idea took hold and became an accepted paradigm for cell-fate determination across biological systems (60). But as we have seen, examination at the level of individual phages indicated that the lysis/lysogeny decision is more accurate than previously assumed—though still not fully deterministic (**Figure 1**, item ❸). In discussing the role of noise, Zeng et al. (12) invoked the concept of hidden variables (originally used in quantum mechanics). This refers to a set of parameters that determine the behavior of the system, but because they are hidden from us, the system appears random rather than deterministic. Exposing these variables will reveal the deterministic nature of the system. In the case of the λ decision, we have only begun to unearth some of these hidden variables: for example, the size of the infected cell (13) and the spatial position of the infecting phage (8, 12). Correspondingly, we have diminished the indeterminacy of the system to only a limited degree. But as highlighted above, additional variables—such as the cell cycle phase of the infected cell—present themselves as excellent candidates to help further reduce the apparent uncertainty of the lysis/lysogeny decision.

To close on the same point with which I began this review, we should remember that the narrative we construct for a system is never full or complete. It is only an approximation valid within a limited range of scales and parameters. And just as classical mechanics, which works so well to explain our everyday experience, fails when tested at the very small (quantum) or very fast (relativistic) limits, so has the textbook picture for the λ life cycle proven insufficient when challenged at the single-cell level. But as they highlight the incompleteness of the existing narrative, the single-cell studies discussed in this review have also begun the formation of a new narrative that is more quantitative and precise. As λ is probed at an increasingly fine resolution, we can look forward to continuing the development of this new narrative.

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