Current Biology, Volume *22*

Supplemental Information

A Single-Molecule Hershey-Chase Experiment

David Van Valen, David Wu, Yi-Ju Chen, Hannah Tuson, Paul Wiggins, and Rob Phillips

Supplemental Inventory

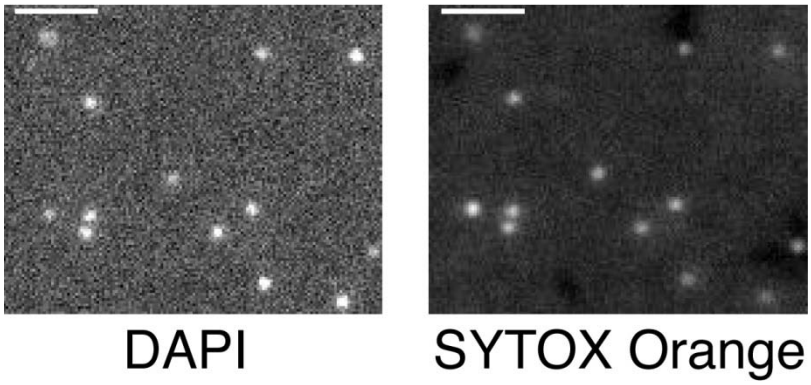
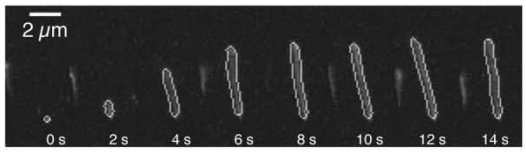


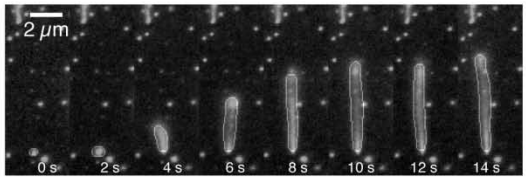
Figure S1. Simultaneous Staining of Phage λ*c*I60 with SYTOX Orange and DAPI, Related to Figures 1 and 2

Here, we verify that SYTOX Orange will penetrate the capsid and stain phage DNA; we compared the SYTOX Orange stain with DAPI, which is known to be a quantitative indicator of DNA mass in phages [1]. Phages were stained with a 1.8 mM concentration of DAPI and 500 nM SYTOX Orange, then flowed into an observation chamber. It has been previously established that DAPI will readily penetrate the phage capsid and stain phage DNA [1, 2]. We observe perfect co-localization of the DAPI and SYTOX Orange signals, demonstrating that SYTOX Orange will enter the phage capsid and stain the phage DNA. Staining and co-localization of the viral DNA with both DAPI and STYOX Orange confirms that SYTOX Orange is both a sensitive and specific indicator for the presence of phage lambda. The presence of fluorescent puncta also provides evidence that SYTOX Orange will not adversely affect phage stability (in contrast to other dyes like SYBR Gold) [3]. The scale bar is 2 microns.

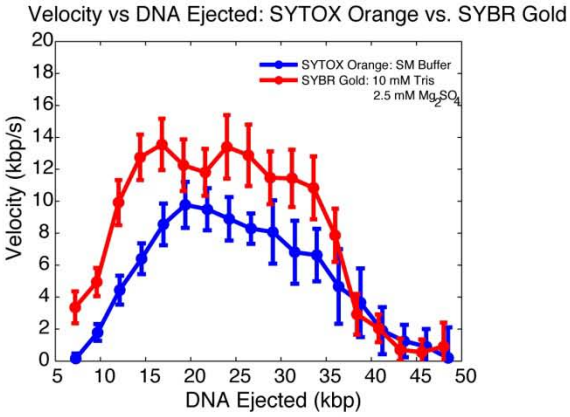
A


2 μm
0 s
2 s
4 s
6 s
8 s
10 s
12 s
14 s

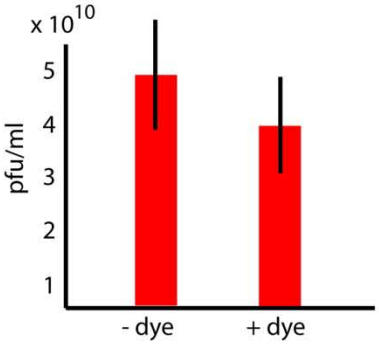
B


2 μm
0 s
2 s
4 s
6 s
8 s
10 s
12 s
14 s

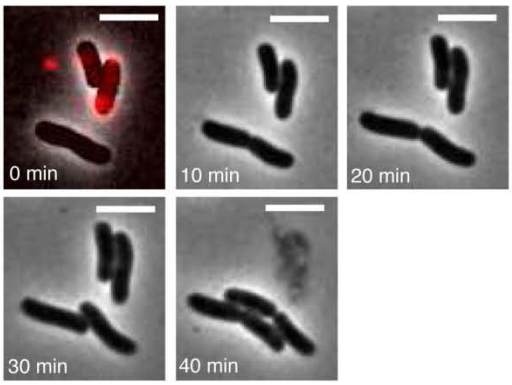
C


Velocity vs DNA Ejected: SYTOX Orange vs. SYBR Gold
SYTOX Orange: SM Buffer
SYBR Gold: 10 mM Tris
2.5 mM Mg_2SO_4
Velocity (kbp/s)
DNA Ejected (kbp)
0
2
4
6
8
10
12
14
16
18
20
5
10
15
20
25
30
35
40
45
50

D


x 10^{10}
pfu/ml
1
2
3
4
5
- dye
+ dye

E


0 min
10 min
20 min
30 min
40 min

F

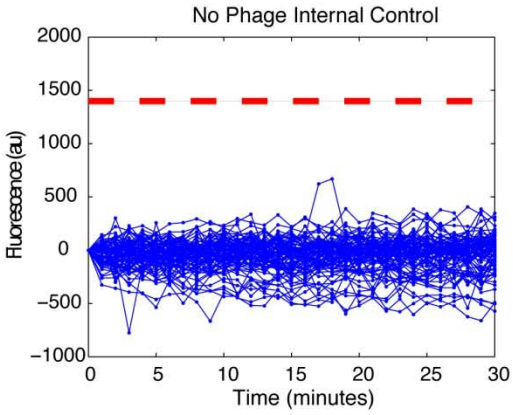

No Phage Internal Control
Fluorescence (au)
Time (minutes)
2000
1500
1000
500
0
−500
−1000
0
5
10
15
20
25
30

Figure S2. SYTOX Orange Penetrates the Phage Capsid, Maintains Phage Stability and Infectivity, Preserves *In Vitro* Ejection Kinetics while Stained, and Does Not Cross the Membrane of Living Cells, Related to Figures 1 and 2

(A and B) Data for the single-molecule *in vitro* DNA ejection experiment. An *in vitro* ejection experiment was performed on unstained phages (A) and pre-stained phages (B) to determine whether the presence of dye inside the capsid has any effect on the ejection dynamics. SYBR Gold was used in the experiment shown in (A) and SYTOX Orange was used in the experiment shown in (B). A MATLAB script was used to segment the DNA; the pixels identified as DNA are outlined.

(C) Velocity vs. the amount of DNA ejected from the capsid for the single-molecule *in vitro* DNA ejection experiment. The presence of dye inside the capsid does not significantly change the underlying dynamics of the ejection process. We hypothesize that difference between the two conditions can be attributed to subtle ionic differences in the buffers for the two experiments. Error bars from standard error (n = 15).

(D) We examined the extent to which these dye molecules alter the macroscopic titers of infectious phage. The unstained phage (- dye) had a titer of 5×10^{10} while the stained phage (5 µM) (+ dye) had a titer of 3.9×10^{10}, a drop of 20%. The experiment was performed in triplicate, and the error bars are from counting error statistics, which our experiments obey. The presence of dye inside the capsid does not have a statistically significant effect on the infectivity of lambda phage in bulk.

(E) A montage demonstrating that cells that have been infected with stained phage will lyse, indicating that presence of the dye neither compromises the infection process nor cellular physiology. SYTOX Orange stained phages are bound to a bacterial cell, placed on an agar pad, and then imaged with fluorescence microscopy over a sufficiently long time to monitor the infection process. Only one fluorescence image was taken to mitigate any possible photo-damage from the excitation of SYTOX Orange, as an oxygen scavenging system was not present. We assume that fluorescence inside a cell signifies that the cell had previously been infected by phage. All of the fluorescent cells that we observed went on to lyse (n=23). Cells with bound phages were also observed; of these, 80% went on to lyse (n=18). This measurement in conjunction with the bulk titering measurement demonstrates that SYTOX Orange does not interfere with the lytic pathway in any substantial way. Scale bar: 4.8 microns.

(F) No phage control. The fluorescence of 89 cells with no phage attached was monitored over the course of a data set. The red line indicates the average change in fluorescence of phages that ejected within the data set. No increase in fluorescence is seen, demonstrating that SYTOX Orange does not cross the membranes of living cells. Moreover, any fluorescent cells we see we assume have been infected.

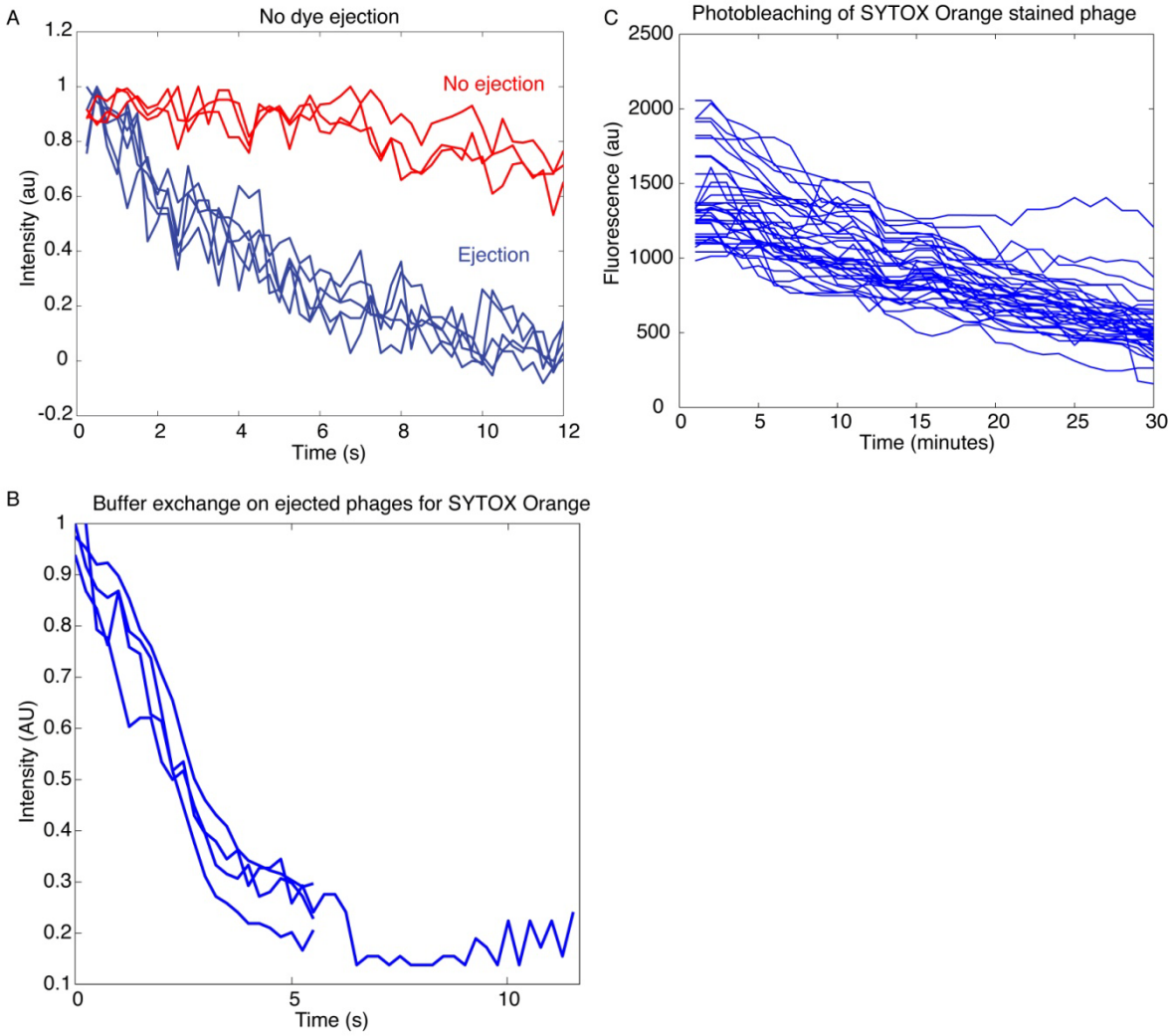

A
No dye ejection
No ejection
Ejection
Intensity (au)
Time (s)
C
Photobleaching of SYTOX Orange stained phage
Fluorescence (au)
Time (minutes)
B
Buffer exchange on ejected phages for SYTOX Orange
Intensity (AU)
Time (s)

Figure S3. Single-Molecule Studies of SYTOX Orange-DNA Unbinding Kinetics and Photobleaching, Related to Figures 1–3

(A) *In vitro* DNA ejection kinetics are not affected by SYTOX Orange staining. Dye leakage from the capsid is much slower than ejections. The phage capsids are rapidly destained as DNA is ejected (blue) in the presence of lambda receptor, LamB, and dye falls off. The kinetics are consistent with *in vitro* ejections [4] in which the ejected DNA was stained, instead of the phage capsid DNA, as is shown here. Compare with phage capsids which did not eject (red) which demonstrates a drop in fluorescence intensity presumably due to photobleaching. The kinetics are markedly different. These ejection curves demonstrate that the *in vitro* kinetics of ejection are unaffected by staining the phage capsid DNA with SYTOX Orange.

(B) Single-molecule *in vitro* DNA ejection in the presence of SYTOX Orange. We performed an *in vitro* ejection experiment with SYTOX Orange instead of SYBR gold following the method in [3] with buffer exchange. Phages affixed to a coverslip are made to eject into a buffered solution containing SYTOX Orange and we wait for the ejection to complete. Then at t = 0 in the figure, the buffer is exchanged with a solution without any dye; as is shown above, the fluorescence signal drops by 70% within 7 seconds. From [5], the k_{off} for SYTOX Orange is 0.58 s^{-1}, and they achieve a 38% reduction in staining with a 100-fold dilution of 500 nM SYTOX Orange within a few seconds, which is consistent with our observations. In our view, these experiments support the interpretation of the *in vivo* ejection assay that when the viral genome enters the cell, the bound dyes fall off and they can then bind onto the much larger host cell genome, allowing us to visualize intracellular fluorescence.

(C) Dye leak and photobleaching of SYTOX Orange stained bacteriophage. In order to properly characterize the ejection events, the combined effect of passive loss of dye from phage and photobleaching must be distinguishable from viral DNA ejection. Phages were stained with SYTOX Orange (as described in the methods section), flown into a KOH cleaned flow chamber, and then allowed to non-specifically adhere to the glass surface. The phages were then imaged with a frame rate of 1 min^{-1}, an exposure time of 500 ms, and with a TRITC filter. These imaging conditions mimic the conditions used to collect most of our data. The trajectories of the total fluorescence above background for 36 phages are shown. The time scale for dye loss and photobleaching is 30 minutes, and all the trajectories are monotonically decreasing. While significant loss of signal does occur over the course of 30 minutes, ejections, as shown in Figures 3D and 4 in the main text, range from 1 minute to 20 minutes. Thus for the typical ejection time scale of 15 minutes, dye loss and photobleaching can account for at most a 30% loss of signal. On the other hand, the fluorescence losses seen for the putative ejection events were much more stereotyped and typically faster than the monotonic decreases seen to result from explicit photobleaching. This lends credence to our use of the rapid decrease in phage signal as a marker for putative *in vivo* ejection events.

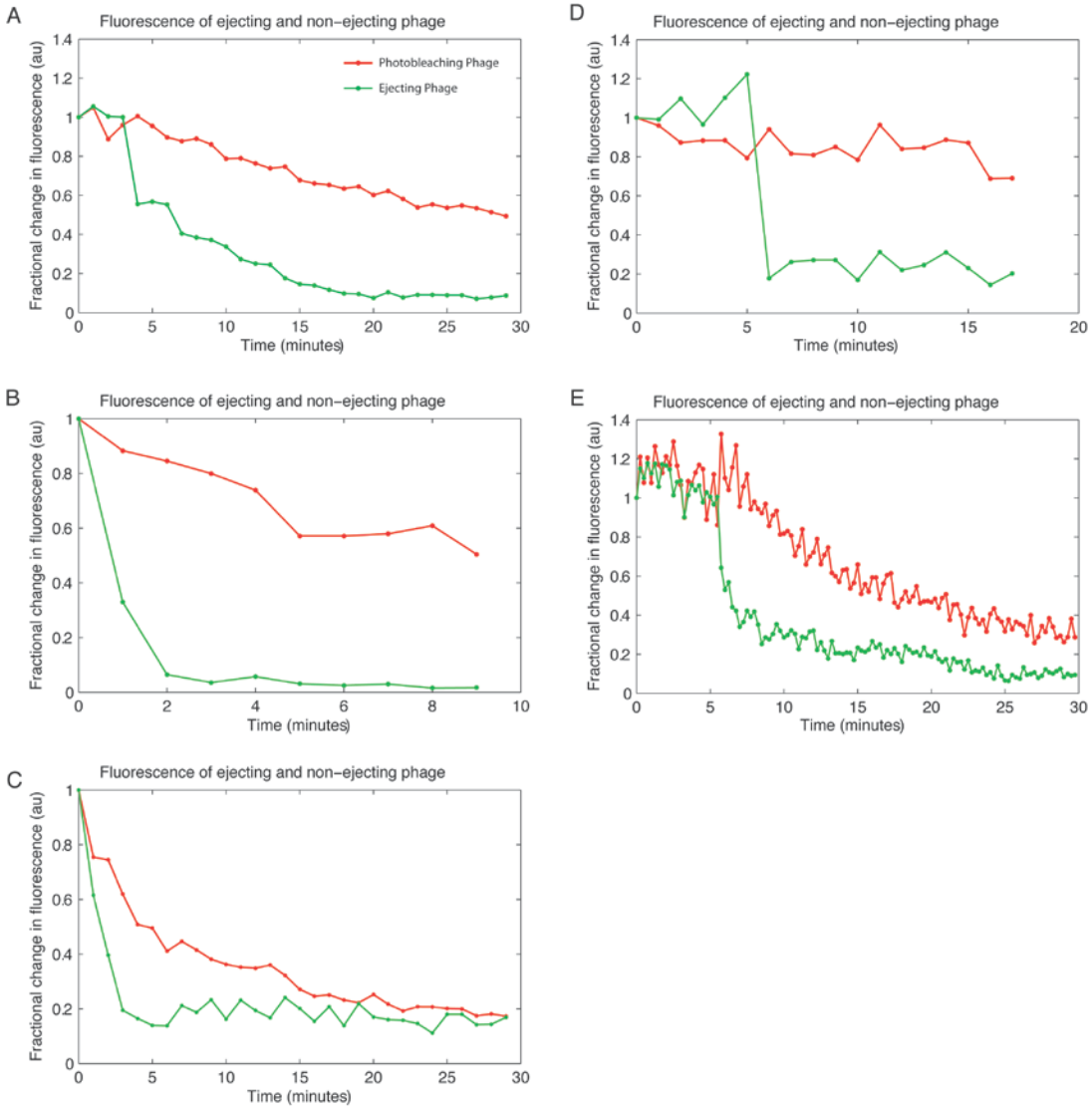


Figure S4. Photobleaching in SYTOX Orange Stained Bacteriophage Bound to Cells, Related to Figures 2 and 3

For those cases in which only one of the bound phages underwent a putative ejection event, by simultaneously monitoring the level of fluorescence in the other, non-ejecting phage, we could directly compare an ejecting and non-ejecting phage, both subject to photobleaching. Some of these phages did not display a significant decrease in fluorescence, indicating that they did not infect the cell. Here we plot the decrease in fluorescence in an infecting phage and a non-infecting phage in five different cells (A-E). The green lines were determined to be ejecting phages, because their fluorescence decrease mirrors the increase in fluorescence inside the cell, and is much faster than photobleaching. The red lines, therefore, represent the fluorescence of non-ejecting phages. The total fluorescence above background for each phage was determined using segmentation masks as demonstrated in Figure 2 in the main text. These values are normalized by the maximum observed fluorescence in each phage to allow for side-by-side comparison of the drop in fluorescence levels. The data demonstrates that the loss in fluorescence in ejecting phages occurs on a faster time scale than photobleaching and dye loss, which allows us to distinguish ejection from photobleaching.

Strains and Media

Here, we detail the stains that were used during this work (Table S1) and recipes for media and buffers (Table S2).

Table S1. Strain and Plasmids that Were Used or Constructed during the Course of Our Study

Strain, Phages, or Plasmid	Description
LE392 (Promega, K9981)	"Wild-type" *E. coli* that carries the *supF* amber suppression phenotype, allowing Sam7 phages to undergo lysis
λcI60 (Gift from Michael Feiss, University of Iowa)	A lambda phage with a clear plaque morphology mutation in the *cI* gene (48.5 kbp genome)
λb221 (Gift from Michael Feiss, University of Iowa)	A lambda phage with a deletion in the b region (37.7 kbp genome)

Table S2. Recipes for Various Media and Buffers Used in Our Study

Media	Description
NZYM (Teknova, N0170)	10g NZ amine, 5g NaCl, 5g Bacto-yeast extract, 2g $MgSO_4$ -7H_2O, 1 L milliQ water, autoclave
LB (EMD Millipore, 71753-5)	10g Bacto-tryptone, 5g yeast extract, 10g NaCl, 1 L milliQ water, autoclave
LBM	LB + 10 mM $MgSO_4$-7H_2O
M9sup	1X M9 salts, 1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$
M9maltose-sup	1X M9 salts, 1 mM thiamine hydrochloride, 0.4% maltose, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$
SM	5.8 g NaCl, 2 g $MgSO_4$-7H_2O, 50 mL 1 M Tris-Cl, pH 7.5, 2 % w/v gelatin (BD, 214340), milliQ water to 1 L, autoclave
TM	50 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$
GODCAT	100 nM glucose oxidase (from *Aspergillus niger*, Sigma, G2133), 1.5 µM catalase (from bovine liver, Sigma, C1345), 500 µL TM

Supplemental Experimental Procedures

Plate Lysis

For a detailed description of components and strains, please see Tables S1 and S2 in the SI. NZYM top agarose (NZYM + 0.7% agarose) and NZYM plates (NZYM + 1.5 % agar (BD, 214010)) were prepared prior to plate lysis. NZYM top agarose was melted on a hot plate and then stored in a 45 ^{o}C water bath until needed. The host cell strain, LE392, was grown up overnight in 5 mL of LB. The saturated cell culture was then centrifuged for 5 minutes at 5,000 g and the pellet was resuspended in 5 mL of SM buffer. A 100 µL aliquot of cells was then mixed with 1 µL of phage stock in a 14 mL culture tube and incubated at 37 ^{o}C for 20 minutes. Next, 3 mL of NZYM top agarose was added to the culture tube, gently mixed, and poured onto a NZYM plate. The plates were incubated for 12--16 hours at 37 ^{o}C or until lysis was visually apparent. After incubation, phages were recovered by pouring 5 mL of SM buffer onto the plate and placing the plate on a rocking station at 4 ^{o}C. After 5 hours, the SM buffer was recovered. The lysate was sterilized by adding chloroform to a concentration of 1% and gently vortexing. Bacterial debris and chloroform were then removed by centrifuging for 10 minutes at 5,000 g; the supernatant was recovered. Plate lysis typically yielded titers of ~ 10^{10} pfu/ml. CsCl purification of phages was performed as in [7].

Real-Time Imaging of DNA Ejection In Vitro

We follow a protocol that was first developed by Mangenot *et al.* and later adapted to use with phage lambda [3, 4, 6]. Microscope coverslips (18x18 mm, #1.5, VWR, 48367-092) were cleaned by sonication in 1M KOH for 10 minutes followed by sonication in water for 10 minutes and dried on a hot plate. Glass slides (75 x 25 mm, VWR, 48300-263) were drilled using a diamond covered drill bit and 5 inches of tubing was attached to the glass slide using epoxy. The flow chamber was assembled using laser cut double-sided adhesive tape (Grace Biolabs, SA-S-1L). A solution of 10^{8} -10^{11} pfu/ml lambda phage was incubated in the assembled flow chambers for 10 minutes. Once focused, the chamber was washed with 200 µL of buffer + 1% oPOE (Alexis Bio-chemicals, 500-002-L005). Buffers were either SM buffer for the SYTOX Orange (Invitrogen, S11368) measurement or 10 mM Tris, pH7.5, 2.5 mM $MgSO_4$. The solution to induce ejection consisted of buffer, 1% oPOE, 1% glucose oxidase/catalase, 1% LamB, 0.5% glucose, 1% beta-mercaptoethanol, and either 10^{-6} diluted SYBR Gold (Invitrogen, S11494) or 500 nM SYTOX Orange. Calibration of lengths and data analysis was performed as in [3]. LamB was extracted from the membranes of *E. coli* pop154 cells [3]; these cells express a *lamB* gene from *S. sonnei*.

Image Analysis

Cells of interest were manually identified in each movie and cropped from the field of view using ImageJ. The phase images for each cell were segmented using custom image analysis software created with MATLAB. Briefly, the phase images from each time point in the movie were registered with the first frame by cross-correlation; this registration was then applied to all fluorescence channels, removing spatial drift from the data set. Next, the registered phase images were all added together; this step greatly reduces difficulty of segmentation. Lastly, thresholding and minor morphological operations were used to create a mask of the cell from the combined image. Cells were occasionally segmented manually when quality phase images were not available. Phage masks were created from the fluorescence channel by a similar process. Quantities of interest, including background levels, and fluorescence intensities inside the cell and phage in each frame were extracted for further analysis.

Supplemental References

[1] Coleman, A. W., Maguire, M. J., and Coleman, J. R. (1981). Mithramycin- and 4'-6-diamidino-2-phenylindole (DAPI)-DNA staining for fluorescence microspectrophotometric measurement of DNA in nuclei, plastids, and virus particles. J. Histochem. Cytochem. *29*, 959–968.

[2] Zeng, L., Skinner, S. O., Zong, C., Sippy, J., Feiss, M., and Golding, I. (2010). Decision making at a subcellular level determines the outcome of bacteriophage infection. Cell *141*, 682–691.

[3] Wu, D., Van Valen, D., Hu, Q., and Phillips, R. (2010). Ion-dependent dynamics of DNA ejections for bacteriophage lambda. Biophys. J. 99, 1101–1109.

[4] Grayson, P., Han, L., Winther, T., and Phillips, R. Real-time observations of single bacteriophage lambda DNA ejections in vitro. Proc. Natl. Acad. Sci. USA *104*,13652–13657.

[5] Yan, X., Habbersett, R., Yoshida, T., Nolan, J., Jett, J., and Marrone, B. (2005). Probing the kinetics of SYTOX orange stain binding to double-stranded DNA with implications for DNA analysis. Anal. Chem. *77*, 3554–3562.

[6] Mangenot, S., Hochrein, M., Radler, J., and Letellier, L. (2005). Real time imaging of DNA ejection from single phage particles. Curr. Biol. *15*, 430–435.

[7] Sambrook, J., and Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.