Chapter 13

Deciphering the Molecular Mechanism of the Bacteriophage φ29 DNA Packaging Motor

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Abstract

The past decade has seen an explosion in the use of single-molecule approaches to study complex biological processes. One such approach—optical trapping—is particularly well suited for investigating molecular motors, a diverse group of macromolecular complexes that convert chemical energy into mechanical work, thus playing key roles in virtually every aspect of cellular life. Here we describe how to use high-resolution optical tweezers to investigate the mechanism of the bacteriophage φ29 DNA packaging motor, a ring-shaped ATPase responsible for genome packing during viral assembly. This system illustrates how to use single-molecule techniques to uncover novel, often unexpected, principles of motor operation.

Key words Single-molecule manipulation, Optical tweezers, Viral DNA packaging, Molecular motor, Ring ATPase

1 Introduction

Many essential cellular processes are driven by nanometer-scale machine-like devices, known as molecular motors, which harness energy from chemical reactions to perform mechanical tasks. Single-molecule manipulation instruments, such as optical tweezers, are ideally suited for studying molecular motors, because the characteristic mechanical parameters of the motor—force, step size, cycle time, etc.—are variables that can be directly measured using these instruments.

One fascinating example of nanomachines is the packaging motor found in double-stranded DNA viruses, including tailed bacteriophages and human pathogens such as herpesviruses [1]. The packaging motor contains multiple ATPase subunits, which form a ring structure and thread viral DNA through the central pore into a preformed protein capsid. The packaging motor of bacteriophage φ29, a small virus that infects *Bacillus subtilis*, is a model system for studying the mechanism of viral packaging and, more generally, the operation of the ubiquitous ring NTPases [2].

These studies have been made possible by the existence of a robust in vitro reconstituted system and extensive biochemical, structural, and single-molecule results. In particular, a series of optical-tweezers-based assays enabled us to tackle fundamental and sophisticated questions regarding the force generation and intersubunit coordination of the φ29 motor [3–8]. The complete mechanochemical model derived from these studies, which precisely describes the coordinated actions of all ATPase subunits (Fig. 1), showcases the power of optical tweezers in elucidating the mechanism and regulation of molecular motors.

In this chapter, we describe protocols for the single-molecule DNA packaging assay with the φ29 motor, which includes bulk activity assessment, microfluidic chamber construction, sample preparation, instrument operation, data acquisition, and data analysis. It is worth noting that the protocols for the φ29 system have been adapted to study other viral packaging motors and ring ATPases, yielding many interesting features of motor dynamics and providing a broad panorama of the diversity of operation of these important cellular machines [9].

2   Materials

2.1   Bulk DNA Packaging Assay

1. Viral components: φ29 proheads and ATPase gp16 (store in small aliquots at −80 °C), φ29 genomic DNA with terminal gp3 protein (DNA-gp3, store at 4 °C). Purified as described in [10]. *See* **Note 1**.
2. 0.025-μm membrane filter (Millipore).
3. 1 M Tris–HCl (pH 7.8).
4. 0.5× TMS buffer: 25 mM Tris–HCl (pH 7.8), 50 mM NaCl, and 5 mM MgCl$_2$.
5. 100 mM ATP. Store at −20 °C.
6. DNase I (Calbiochem).
7. 0.5 M EDTA.
8. Proteinase K (New England Biolabs).

2.2   DNA Preparation for Single-Molecule Packaging Assay

1. DNA-gp3 (see above).
2. 1 M Tris–HCl (pH 7.8).
3. Selected restriction enzyme ClaI, XbaI, BstEII, or NcoI with its respective 10× buffer (New England Biolabs).
4. Klenow fragment exo$^−$ (New England Biolabs).
5. Biotinylated deoxyribonucleotides (Invitrogen).
6. T4 DNA ligase and 10× T4 ligase buffer (New England Biolabs).
7. 100 mM ATP.
8. PCR thermocycler.
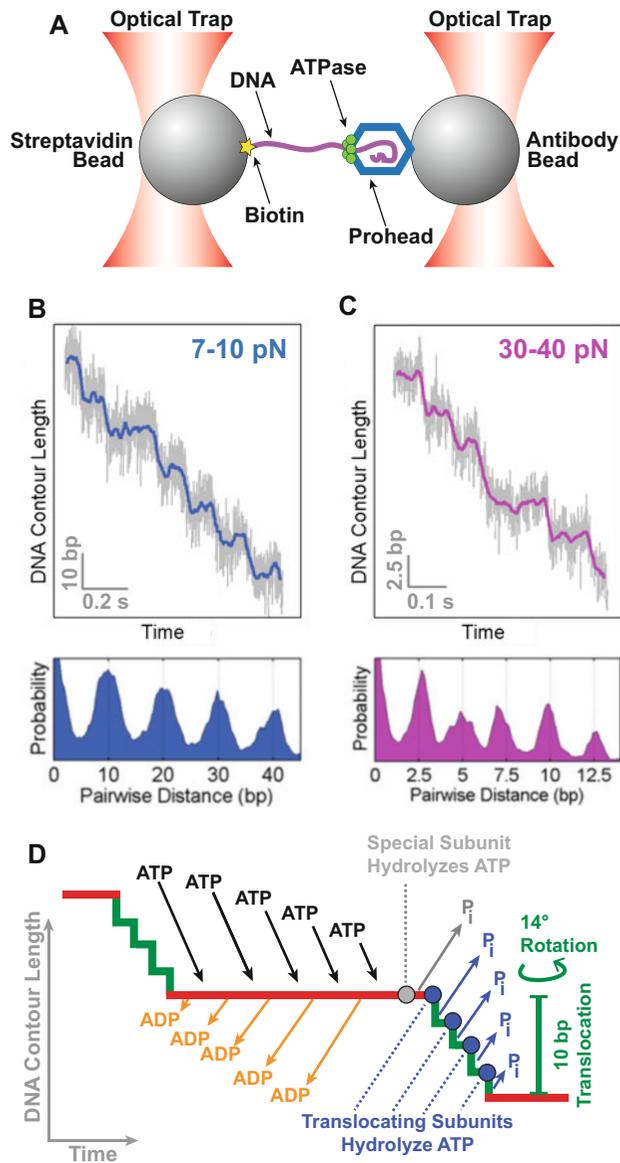
Fig. 1 Single-molecule DNA packaging assay with the φ29 motor. (**a**) Geometry of the dual-trap optical tweezers experiment. (**b**) (*Top*) A sample packaging trace collected at an external force of 7–10 pN. (*Bottom*) The corresponding pairwise distance distribution (PWD) demonstrates that DNA is translocated in 10-bp bursts in this force regime. 2500-Hz raw data are shown in *gray* and 250-Hz filtered data in *blue*. (**c**) A sample packaging trace collected at an external force of 30–40 pN (*Top*) and its corresponding PWD (*Bottom*). In this force regime the 10-bp bursts break up into four 2.5-bp steps. (**d**) Mechanochemical model of the φ29-packaging motor. ADP release and ATP binding events in the five ATPase subunits occur in an interlaced fashion during the dwell phase (*red*). One of the five subunits is "special" in that its hydrolysis and inorganic phosphate release do not result in DNA translocation, but rather signal the other four subunits to hydrolyze ATP, release P_i, and each translocate 2.5 bp of DNA during the burst phase (*green*). In each cycle, 10-bp DNA translocation is accompanied by a 14° DNA rotation relative to the motor. This rotation ensures that the DNA makes specific contacts with the same special subunit in the next cycle. Adapted from refs. [7] and [8] with permission from Elsevier

2.3 Bead Preparation	1. 1× phosphate buffered saline (PBS buffer).
	2. 0.5× TMS buffer (see above).
	3. Polystyrene beads stock solution: 1 % (w/v) 0.88-μm Protein G-coated beads, 1 % (w/v) 0.90-μm streptavidin-coated beads (Spherotech). Store at 4 °C.
	4. Vortexer.
	5. Rotator.
	6. Anti-phage antibodies stock solution (1 mg/mL) (produced by the Jardine and Grimes Lab, University of Minnesota). Store in 20 μL aliquots at −80 °C.
2.4 Microfluidic Chamber Construction	1. Cover glass (VWR, size #1, 24 × 60 mm).
	2. Nescofilm (Karlan).
	3. Laser cutter (Universal Laser Systems).
	4. Glass dispenser tube (King Precision Glass, 0.1-mm diameter).
	5. Heat block (100 °C).
	6. PE20 polyethylene tubing (BD Intramedic).
2.5 Single-Molecule Packaging Assay	1. 10× TMS buffer: 500 mM Tris–HCl (pH 7.8), 1 M NaCl, and 100 mM MgCl$_2$.
	2. BSA (20 mg/mL) (New England Biolabs).
	3. RNaseOUT (40 units/μL) (Invitrogen).
	4. 100 mM ATP.
	5. 100 mM ATPγS (Sigma-Aldrich).
	6. Oxygen-scavenging system: 100 μg/mL glucose oxidase, 20 μg/mL catalase, and 5 mg/mL dextrose (Sigma-Aldrich).
	7. ApaLI (10 units/μL) (New England Biolabs).
	8. 1-mL syringes (BD).
	9. Needles (BD PrecisionGlide, 26 G × ½ in.).
	10. High-resolution dual-trap optical tweezers. *See* **Note 2**.
2.6 Data Analysis	1. Custom LabView software.
	2. Custom MATLAB software.

3 Methods

3.1 Bulk DNA Packaging Assay	The in vitro DNA packaging activity of the motor is evaluated by measuring the amount of packaged DNA inside the phage capsid that is resistant to DNase digestion [10].
	1. DNA-gp3, isolated from phage-infected *B. subtilis* cells or purified phages, is dialyzed on a 0.025-μm membrane filter against 10 mM Tris–HCl (pH 7.8) for 45 min.

2. φ29 proheads $(1 \times 10^{11}$ copies) are mixed with DNA-gp3 $(5 \times 10^{10}$ copies) and gp16 $[(1.2–1.5) \times 10^{12}$ copies] in $0.5\times$ TMS buffer in a total volume of 18 µL. The mixture is incubated for 5 min at room temperature.

3. Add 2 µL of 5 mM ATP and incubate the mixture for 15 min at room temperature.

4. Unpackaged DNA is digested by adding DNase I to 1 µg/mL. Incubate for 10 min at room temperature.

5. To deactivate DNase I and release the packaged DNA from viral capsids, the mixture is treated with 25 mM EDTA (final concentration) and 500 µg/mL Proteinase K (final concentration) for 30 min at 65 °C.

6. DNA packaging efficiency is evaluated by running a 1 % agarose gel.

3.2 DNA Preparation for Single-Molecule Packaging Assay

The φ29 genomic DNA is 19.3-kbp in length, with one copy of the terminal protein gp3 covalently bound to each 5′ terminus. To systematically investigate the effect of capsid filling level on the motor's packaging behavior, we use DNA substrates of various lengths in the single-molecule packaging assay (Table 1) [8].

1. After dialysis in 10 mM Tris–HCl, DNA-gp3 is digested with one selected restriction enzyme (Table 1). Use 1 unit of enzyme to digest 1 µg of DNA-gp3 for 1 h. Choose the optimal buffer and temperature according to the manufacturer's protocol.

2. The 5′ overhang from the restriction cut is filled in with biotinylated nucleotides using the Klenow fragment of DNA polymerase I (exo⁻ mutant). Use 1 unit of Klenow fragment and 100 pmol of nucleotides for every 1 µg of DNA-gp3. Set the reaction at 37 °C for 30 min, then 75 °C for 15 min to deactivate the enzyme.

3. Dialyze the solution on a 0.025-µm membrane filter against 10 mM Tris–HCl (pH 7.8) for 45 min. Store the DNA substrate at 4 °C. *See* **Note 3**.

Table 1
Summary of the different DNA lengths used in the single-molecule packaging experiments

Capsid filling level (%)	DNA length (bp)	Restriction enzyme used	Remaining overhang
32	6147	ClaI	5′ CG
46	8929	XbaI	5′ CTAG
65	12,466	BstEII	5′ GTCAC
78	15,023	NcoI	5′ CATG

4. To generate DNA substrates longer than the φ29 genome length, a biotinylated DNA piece is ligated to the enzyme-digested DNA-gp3. For example, to create a 21-kbp DNA substrate, first generate a 6-kbp DNA piece that is PCR amplified from lambda DNA using a biotinylated primer and cut with NcoI; then ligate it to NcoI-cut DNA-gp3. Use 5× molar excess of 6-kbp DNA to DNA-gp3. Use New England Biolabs' standard T4 DNA ligase protocol.

5. After the ligation reaction, dialyze the mixture in 10 mM Tris–HCl (pH 7.8) for 45 min. Store the product at 4 °C.

3.3 Bead Preparation

In the single-molecule experiment, a prohead-ATPase-DNA complex is tethered between two beads held in two laser traps (Fig. 1a). The viral capsid is attached to a bead coated with antibodies against the major capsid protein gp8. The biotinylated distal end of the DNA substrate is attached to a streptavidin-coated bead.

3.3.1 Preparing Antibody-Coated Beads

1. Pipette 40 μL of 1 % (w/v) 0.88-μm Protein G-coated beads in a 1.5-mL microcentrifuge tube.

2. Add 1× PBS buffer to a total volume of 200 μL.

3. Resuspend the beads by vortexing the solution on high speed for 30 s. Spin the beads down at $10,000 \times g$ for 2 min in a benchtop centrifuge.

4. Remove the supernatant.

5. Repeat **steps 2–4** twice.

6. Resuspend the pellet in 30 μL of 1× PBS buffer.

7. Add 20 μL of anti-phage antibodies (1 mg/mL; purified from rabbit antisera prepared against φ29 proheads). Gently tumble the mixture for 4 h in a tube rotator at room temperature.

8. Wash the beads by repeating **steps 2–4** three times.

9. Resuspend the beads in 60 μL of 1× PBS buffer. Store at 4 °C.

3.3.2 Preparing Streptavidin-Coated Beads

1. Pipette 30 μL of 1 % (w/v) 0.90-μm streptavidin-coated beads in a 1.5-mL microcentrifuge tube.

2. Add 0.5× TMS buffer to a total volume of 200 μL.

3. Resuspend the beads by vortexing the solution on high speed for 30 s. Spin the beads down at $10,000 \times g$ for 2 min in a benchtop centrifuge.

4. Remove the supernatant.

5. Repeat **steps 2–4** twice.

6. Resuspend the pellet in 60 μL of 0.5× TMS buffer. Store at 4 °C.

3.4 Microfluidic Chamber Construction

The design of the microfluidic chamber is shown in Fig. 2.

1. Drill six holes on a cover glass using a laser cutter.

2. Make a three-channel pattern on a piece of Nescofilm using a laser cutter.

3. Lay the patterned Nescofilm on a second cover glass. Use two glass dispenser tubes to connect the channels. Then put the drilled cover glass on top of the Necsofilm.

4. Put the chamber on a 100 °C heat block for 30 s. Gently press the chamber to seal the two cover glasses. Inspect for any air bubbles.

5. Mount the chamber onto a metal frame. Assemble the inlet/outlet tubings. Then place the chamber between the two objectives of the optical tweezers instrument.

6. Wash the channels with 1 mL of water and then 1 mL of $0.5\times$ TMS buffer before each experiment.

3.5 Single-Molecule Packaging Assay

DNA packaging is initiated in a 1.5-mL microcentrifuge tube by feeding DNA substrates to reconstituted prohead/ATPase complexes in the presence of ATP. Packaging is then stalled by adding the non-hydrolyzable analog ATPγS. The stalled packaging complexes are delivered to the microfluidic chamber and restarted in an ATP-containing solution. *See* **Note 4**.

3.5.1 Bead Passivation

1. Add 2 μL of stock streptavidin-coated or antibody-coated beads and 1 μL of 20 mg/mL BSA to 20 μL of $0.5\times$ TMS buffer.

2. Vortex at high speed for 45 min at room temperature. Then put the beads on ice.

3. Unused passivated beads are stored at 4 °C. Vortex again before using them the next day.

3.5.2 Assembling Stalled Packaging Complexes

1. Add in order: 4.5 μL of H_2O, 1 μL of $10\times$ TMS buffer, 0.5 μL of RNaseOUT, 2 μL of biotinylated DNA-gp3 (2.5×10^{10} copies), and 4 μL of proheads (1×10^{11} copies). Mix gently.

2. Add 4 μL of gp16 (2.5×10^{12} copies). Mix gently. Incubate the mixture for 5 min at room temperature.

3. Packaging is initiated by adding 2 μL of 5 mM ATP. Mix well and incubate for 30 s.

4. Packaging is stalled by adding 2 μL of 5 mM ATPγS. Mix well.

5. The stalled complexes are stored on ice and must be used within the same day. *See* **Note 5**.
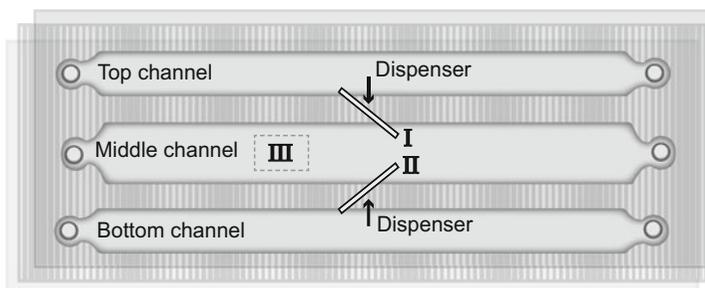
Fig. 2 Microfluidic chamber design. The solutions flow from *left* to *right*

3.5.3 Making Solutions for the Three Channels of the Microfluidic Chamber (Fig. 2)

1. Top channel solution. 4 μL of passivated streptavidin-coated beads are diluted in 1 mL of 0.5× TMS buffer.

2. Middle channel solution. 1 mL of 0.5× TMS buffer supplemented with the oxygen scavenger system (to prevent the formation of reactive singlet oxygen that would damage the tether) and ATP. A typical saturating ATP concentration is 250 μM.

3. Bottom channel solution. Mix in order: 10 μL of 0.5× TMS buffer, 2 μL of 5 mM ATP, 2 μL of 5 mM ATPγS, 0.5 μL of RNaseOUT, 0.5 μL of ApaLI, 4 μL of passivated antibody-coated beads, and 1 μL of stalled complexes. Incubate for 20 min at room temperature. Then dilute the mixture in 1 mL of 0.5× TMS buffer containing 100 μM ATP and 100 μM ATPγS. *See* **Note 6**.

3.5.4 Forming Tethers and Recording Packaging Trajectories

1. Transfer the solutions above from 1.5-mL microcentrifuge tubes to 1-mL syringes. Connect the syringes to the inlet tubings of the microfluidic chamber via 26G½ needles.

2. Push ~50 μL of the top channel solution into the top channel. Streptavidin-coated beads are delivered to Position I of the middle channel through the dispenser tube (Fig. 2). Catch a single bead in Trap A (Fig. 1a, left).

3. Push ~50 μL of the bottom channel solution into the bottom channel. Antibody-coated beads, which are conjugated to stalled complexes, are delivered to Position II of the middle channel through the dispenser tube. Catch a single bead in Trap B (Fig. 1a, right).

4. Bring Trap A and Trap B close to each other, while quickly moving them to Position III (within 10 s). *See* **Note 7**.

5. Move the two traps apart. If the force reading increases as the traps are being separated, it is an indication that a tether has formed. *See* **Note 8**.

6. Start recording the positions of the two beads and the trap separation at 2.5-kHz bandwidth. *See* **Note 9**.

7. The packaging experiment is typically conducted in a semi-passive mode, in which the distance between the two optical traps is adjusted periodically so that the force applied to the motor is kept within a small range (e.g., 7–10 pN). *See* **Note 10**.

3.6 Data Analysis

1. Trap stiffness and detector response are calibrated by fitting a modified Lorentzian to the fluctuation power spectrum of a trapped bead [11, 12].

2. The optical force F is determined by $F = kd$, where k is the trap stiffness, and d is the displacement of the bead from the trap center achieved by back focal plane interferometry at the position-sensitive photodetectors.

3. The extension of the tether is calculated by subtracting the bead displacements and the bead radii from the trap separation.

4. The DNA tether's contour length is calculated from the measured force and tether extension using the worm-like chain model of double-stranded DNA elasticity [13], using a persistence length of 53 nm and a stretch modulus of 1200 pN. Length in nm is then converted to base pairs (bp) using an average B-form DNA rise of 0.34 nm/bp.

5. Raw 2.5-kHz data are filtered to 100–250 Hz for further analysis. A modified Schwarz Information Criterion (SIC) algorithm is used to find steps in the packaging traces (Fig. 3). *See* **Notes 11** and **12**.

4   Notes

1. φ29-like phages have a unique and essential RNA component, known as the prohead RNA (pRNA), in their packaging motor complexes. Some experiments involve the usage of truncated or mutated versions of pRNA. In these cases, purified proheads are first treated with RNase to remove the wild-type pRNA. These RNA-free proheads are then incubated with fresh pRNA molecules prior to use.

2. The single-molecule packaging experiments are conducted on a home-built high-resolution dual-trap optical tweezers instrument. Detailed information on the concept, design, and use of this instrument can be found in [14].

3. Despite the fact that two biotinylated DNA-gp3 species are generated by this procedure, it was shown that the left end of the φ29 genome is preferentially packaged into the prohead
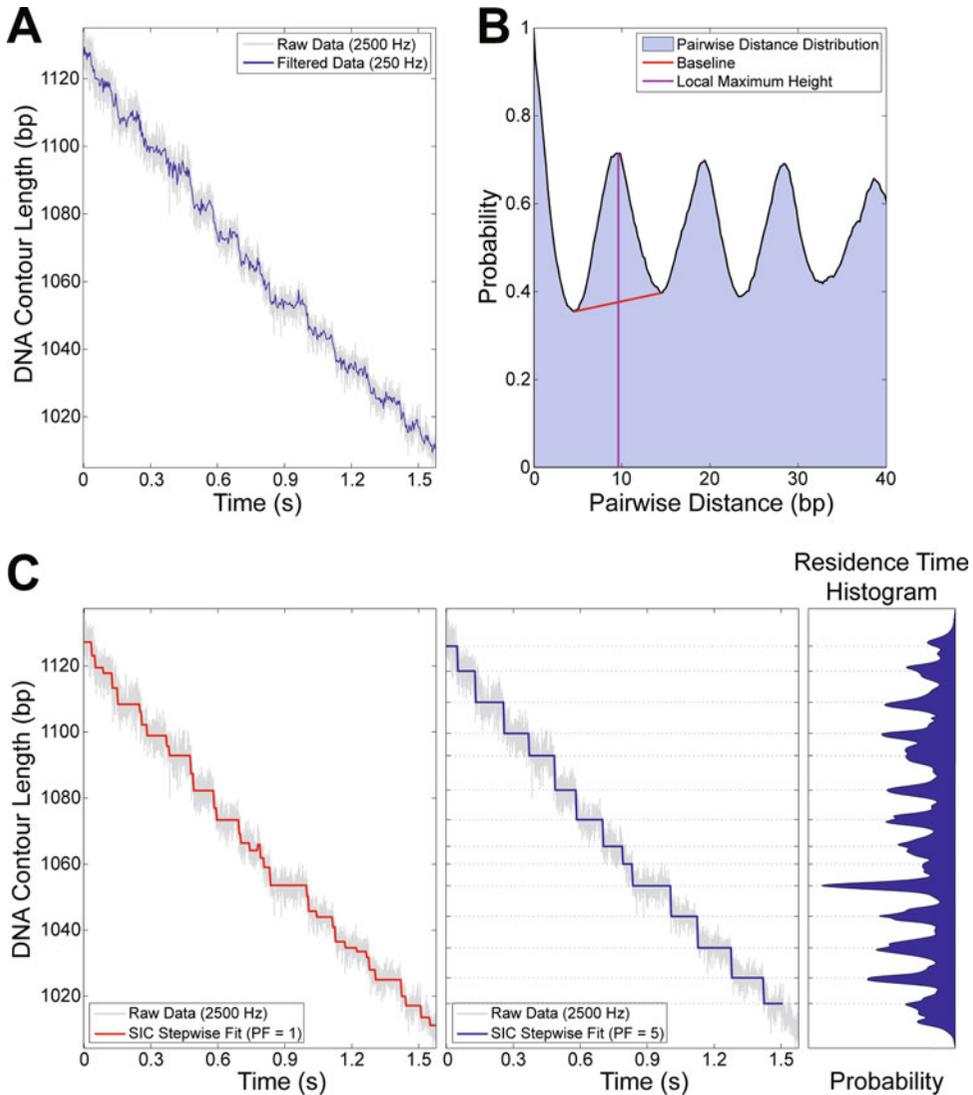
Fig. 3 Step finding in single-molecule packaging traces. (**a**) A sample packaging trace shown in the 2500-Hz raw form (*gray*) and the 250-Hz filtered form (*blue*). (**b**) PWD for the trace shown in Panel (**a**). The PWD plot contains peaks at integer multiples of 10 bp. We define PWD contrast as the height of the local maximum divided by the baseline, which is determined by the two nearest local minima. Traces with a PWD contrast larger than 1.2 typically exhibit clear stepping and are used for further dwell-time analysis. (**c**) (*Left*) The SIC step-finding algorithm in its original form [17] over-fits the trace from Panel (**a**). (*Middle*) A modified SIC algorithm with a penalty factor of 5 yields a more reasonable stepwise fit (*see* **Note** 11). 250-Hz filtered data are used for step finding. (*Right*) To construct a residence-time histogram, each filtered point is represented as a Gaussian function centered at the mean of the data, with a width equal to the local standard error of the unfiltered data. The residence-time histogram is the sum of the Gaussian functions of all filtered data points. The local maxima in the residence-time histogram coincide with the dwells identified by the modified SIC algorithm, thus validating this method. Reproduced from ref. [7] with permission from Elsevier

(pRNA likely plays a key role in such selection) [15]. Therefore, it is not necessary to separate these two species before mixing them with the proheads in a single-molecule packaging experiment.

4. Packaging can also be initiated in situ without prepackaging and stalling in the tube [6, 16]. In this case, the biotinylated DNA is bound to a streptavidin-coated bead, and the prohead/ATPase complex is attached to an antibody-coated bead. Packaging is then initiated by bringing the two beads into close contact in the presence of ATP. This procedure allows for the detection of very early stages of packaging.

5. The quality of the stalled complexes is essential for the outcome of the single-molecule packaging experiment. Once prepared, the stalled complexes can be used for the entire day. However, we notice that the efficiency of forming active tethers slowly drops with time, perhaps due to residual packaging and/or disassembly of the stalled complexes in the tube. Thus it is advised to prepare a fresh stalled complex sample every 4–5 h.

6. The ApaLI cutting site is located near the left end of the φ29 genome (214 bp from the left terminus). DNA is protected from ApaLI cleavage if packaging is properly initiated. Therefore we add ApaLI to the mixture in order to avoid tethering with inactive prohead/ATPase/DNA complexes that did not initiate packaging.

7. Recording of DNA packaging activity is performed at Position III, away from the dispenser tubes opposite to the direction of flow. This is to avoid accidentally capturing additional beads into the traps during data collection. This region also has reduced buffer turbulence, which helps lower data noise.

8. The likelihood of forming a tether is dependent on the density of stalled complexes on the bead. Too high a density causes multiple tethers between the bead pair, whereas too low a density makes experiments time-consuming. We empirically adjust the ratio of bead concentration to stalled complex concentration, such that on average one tether forms every three to four bead pairs. Under this condition most tethers are single tethers, which is desired.

9. During data recording, we sample the voltages proportional to the position of the light centroid in x and y directions at the two position-sensitive photodetectors, and the voltage proportional to the total amount of light at each detector. We also record the voltages proportional to the horizontal and vertical angle of the piezo mirror that controls the position of the steerable trap. These eight voltage signals are acquired at a rate of 500 kHz, or 62.5 kHz per channel. They are then averaged to 2.5-kHz bandwidth before saving.

10. After the packaging process has completed, the tether is intentionally broken by applying a high force (~**30 pN**). Two additional calibration files are collected with the same bead pair. First, the positions of the two beads are recorded at 62.5 kHz to determine their fluctuation power spectra. Second, the two beads (untethered) are slowly brought together and the voltage signal as a function of trap separation is recorded. Residual force calculated from this baseline signal is subtracted from the force measured during packaging to correct for the interference pattern between the two traps.

11. The SIC algorithm is an iterative procedure that fits a series of steps to the data and assesses the fit quality for every round of fitting. In the original algorithm [17], the quality of the fit is determined via the formula $\mathrm{SIC}(j_1, \ldots, j_k) = (k+2)$ $\log(n) + n\log\left(\hat{\sigma}_{j_1,\ldots,j_k}^2\right)$, where n is the number of data points, k is the number of steps, and $\hat{\sigma}_{j_1,\ldots,j_k}^2$ is the maximum likelihood estimator of variance when k steps are fitted to the data. We find that the original SIC algorithm over-fits experimental data containing colored noise (Fig. 3). We therefore introduce an additional penalty factor (PF): $\mathrm{SIC}(j_1, \ldots, j_k) = \mathrm{PF}(k+2)$ $\log(n) + n\log\left(\hat{\sigma}_{j_1,\ldots,j_k}^2\right)$ [7]. Optimal stepwise fits can usually be achieved using PF values of 3–5. Steps assigned by this method are validated using a residence time histogram analysis (Fig. 3).

12. Optimal resolution of an optical tweezers measurement is achieved when the DNA tether length is less than 2 kbp, since longer tethers are intrinsically noisier. Thus, in order to probe the stepping behavior of the motor at different capsid filling levels, DNA substrates of various lengths are used (Table 1).

Acknowledgments

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