Chapter 21

Methods for Single-Molecule Sensing and Detection Using Bacteriophage Phi29 DNA Packaging Motor

Farzin Haque, Hui Zhang, Shaoying Wang, Chun-Li Chang, Cagri Savran, and Peixuan Guo

Abstract

Bacteriophage phi29 DNA packaging motor consists of a dodecameric portal channel protein complex termed connector that allows transportation of genomic dsDNA and a hexameric packaging RNA (pRNA) ring to gear the motor. The elegant design of the portal protein has facilitated its applications for real-time single-molecule detection of biopolymers and chemicals with high sensitivity and selectivity. The robust self-assembly property of the pRNA has enabled biophysical studies of the motor complex to determine the stoichiometry and structure/folding of the pRNA at single-molecule level. This chapter focuses on biophysical and analytical methods for studying the phi29 motor components at the single-molecule level, such as single channel conductance assays of membrane-embedded connectors; single molecule photobleaching (SMPB) assay for determining the stoichiometry of phi29 motor components; fluorescence resonance energy transfer (FRET) assay for determining the structure and folding of pRNA; atomic force microscopy (AFM) for imaging pRNA nanoparticles of various size, shape, and stoichiometry; and bright-field microscopy with magnetomechanical system for direct visualization of viral DNA packaging process. The phi29 system with explicit engineering capability has incredible potentials for diverse applications in nanotechnology and nanomedicine including, but not limited to, DNA sequencing, drug delivery to diseased cells, environmental surveillance, and early disease diagnosis.

Key words phi29 DNA packaging motor, Nanomedicine, RNA nanotechnology, Nanopore, Conductance, RNA nanoparticle, pRNA, Photobleaching, FRET, AFM

1 Introduction

Phi29 bacteriophage is a dsDNA virus [1, 2] that packages its genome into a preformed protein shell, termed procapsid using an ATP driven DNA packaging motor [3, 4]. This biomotor (30 nm in dimension) first constructed in vitro three decades ago [5] is one of the most well studied motor system and has been shown to be one of the most powerful biomotors studied to date capable of generating forces of up to 110 pN [6]. The phi29 motor consists of three major structural components (Fig. 1a): a central dodecameric portal channel, termed connector that serves as a
corridor for transporting genomic dsDNA into the procapsid during the packaging process and subsequent ejection into bacterial host cell for infection [7, 8] (Fig. 1b); a hexameric packaging RNA (pRNA) ring [9–12] (Fig. 1c) that gears the motor; and a hexameric ring of ATPase gp16 [13, 14] that provides energy by hydrolyzing ATP. The elegant design of the motor components has inspired their utility as versatile building blocks for bottom-up self-assembly of nanostructures for diverse applications in nanotechnology and synthetic biology. Herein we outline detailed electrophysiological assays for using the connector for single molecule detection of chemicals and biopolymers, and single molecule fluorescence and biophysical assays for elucidating the structure and stoichiometry of motor RNA components as well as for investigating the mechanical properties of the biomotor.

A key step for viral reproduction is genome packaging, which is accomplished by a biomotor using ATP. Previously, biomotors were typically classified into linear and rotation motors [15]. For over three decades, it was widely believed that the DNA packaging motors of dsDNA viruses are rotation motors [3, 4, 16–20]. However, extensive studies showed that both the motor channel and the dsDNA did not rotate during motor action [11, 21–25]. Furthermore, the swivel structure of the phi29 motor channel and the dsDNA helix were arranged in an opposite and antiparallel orientation [26], which is a puzzle since the rotation mechanism by bolt

Fig. 1 (a) Structure of phi29 DNA packaging motor showing dodecameric connector, hexameric pRNA, and hexameric ATPase. (b) Side and top views of the connector showing acidic (red), basic (blue), and neutral (white) amino acids, and pore dimensions. (c) Side and top views of hexameric pRNA. Figures reproduced with permissions from: (a) ref. 28 © BioMed Central Ltd.; (b) ref. 37 © Macmillan Publishers Ltd.; (c) ref. 25 © Elsevier
and screw nut threading requires that both threads of the bolt and nut are in the same orientation. Recently, we discovered a new class of biomotors that uses a revolution mechanism without any rotation, coiling, friction, or torque force (Fig. 2) [14, 25, 27]. This finding resolves all puzzles introduced throughout the history of painstaking studies that have quelled fervent debates concerning the structure and function of the viral DNA packaging motors. This revolution motion mechanism has subsequently been found to be widespread in different biological systems [28]. Two simple parameters, chirality and channel size can be used to distinguish the rotation motors from revolution motors [28] (Fig. 2b, c).

Nanopore based sensing has emerged as an attractive and versatile platform in recent years for myriad applications [29–36]. The principle is based on electrical detection of analytes as it interacts with the nanosized pore embedded in a substrate under an applied potential. It is label-free, amplification-free, needs ultralow sample amounts and is a simple, cheap, and rapid detection process requiring no special expertise. This platform can be easily translated into a real-time, high-throughput multiplexed detection tool. We have developed methods for inserting the reengineered phi29 connector into lipid membranes to serve as a membrane-embedded nanopore [37]. This is the first viral protein channel that is neither a membrane protein nor an ion channel that has been reconstituted into a lipid bilayer. With a pore size of 3.6 nm at the narrowest constriction, the connector is also the largest biological nanopore studied to date. The resulting system has been shown to be robust under a wide range of solution conductances [38] and generates extremely sensitive and distinct fingerprints for characterizing analytes at ultralow concentrations [20, 26, 28, 37–42]. Modifications at the terminal ends and chemical conjugations within the large pore cavity can be made with relative ease for added functionality [43, 44]. The electrophysiological assays described here can be used for sensing and detection of a wide range of analytes for target applications in diagnosing diseases at asymptomatic stages, monitoring disease stages/progession, surveying the environment, sequencing DNA, RNA, and even peptides, studying the mechanisms of viral DNA packaging, and biomimetic applications such as stimuli-responsive switches and biomemory devices. These methods can be further applied to utilize other phage portal channels from SPP1, T3, T4, T5, and T7, each with characteristic sizes and shapes desirable for customized applications [45].

DNA, RNA, and proteins have defined nanoscale features suitable for constructing molecular architectures at the nanometer scale with defined size, shape and stoichiometry. We have applied bottom-up self-assembly technique of RNA nanotechnology to construct varieties of thermodynamically stable pRNA-based nanoparticles in a controllable and predictable manner using three toolkits [10, 46–52]: loop–loop interactions; foot-to-foot
Fig. 2 (a) An illustration depicting motors of different categories: Linear motor, rotation motor, and revolving motor. (b, c) Differences between revolving motor and rotation motor based on their channel chirality (b) and
palindrome sequence mediated interactions; and branch extension based on three-way junction motif. Among different topographic imaging methods, atomic force microscopy (AFM) is an attractive approach [53] that has enabled us to verify whether the arrangements of the building blocks in RNA nanoparticles are indeed consistent with their predicted designs [46–52]. AFM can also be applied for dynamic imaging of RNA nanostructures as well as force spectroscopy studies for investigating RNA/RNA intermolecular and intramolecular interactions [54].

Determining the stoichiometry, size and arrangements of macromolecules within biological complexes is an arduous challenge. Conventional optical microscopy is limited by diffraction, typically 200 nm for visible light. Recent developments in super high resolution microscopy methods, such as photoactivated localization microscopy (PALM) [55], stochastic optical reconstruction microscopy (STORM) [56], and stimulated emission depletion (STED) [57] have enabled biological samples to be imaged with nanometer precision [55, 58–61]. We developed a simple and straightforward method called single molecule photobleaching (SMPB) for direct counting of pRNA subunits on the phi29 motor [11, 62] and for elucidating the mechanism of protein–RNA interactions [63]. The principle is based on photobleaching a single fluorophore present on the molecule of interest, which results in a quantized drop in fluorescence intensity, measured using single molecule total internal reflection (TIRF) microscopy. Using TIRF setup, single fluorophores can be reliably detected from background fluorescence in the bulk solution. Based on the stepwise intensity drops over time, the number of fluorophores within a diffraction limited spot can be counted to reveal the stoichiometry of the motor components. This technique can be readily applied for single molecule counting of DNA, RNA, and proteins in a variety of multisubunit biocomplexes and nanostructures.

Elucidating the structure and folding of macromolecules or determining the conformational changes of biomolecules in response to stimulus (such as ligand binding or ions) requires imaging systems with nanometer-scale resolution. We have developed a method using single molecule fluorescence resonance energy transfer (smFRET) for determining the distances between RNA fragments. The principle is based on energy transfer between

---

**Fig. 2** (continued) channel size (c). The channel in revolving motors has an antichiral configuration with the right-handed dsDNA, while the channel in rotation motors has the same chirality with the dsDNA. Revolving motors usually have channels larger than 3 nm in diameter, while rotation motors have smaller channels (<2 nm in diameter) to ensure full contacts with dsDNA chains. Figures reproduced with permissions from: (a) ref. 15 © American Society for Microbiology; ref. 28 © BioMed Central Ltd.; ref. 83 © Elsevier; (b, c) ref. 28 © BioMed Central Ltd.
a donor fluorophore (e.g., Cy3) and acceptor fluorophore (e.g., Cy5) within a distance of 10 nm [64]. Based on the efficiency of energy transfer, the distance between the FRET pairs can be deduced following the Förster theory [65–67]. The method can be applied to study conformational changes of RNA motifs, kinetics of ribozyme-mediated catalysis, structural analysis of RNA and DNA nanoparticles, and so forth.

Finally, we outline methods for studying the phi29 motor DNA packaging process at the single molecule level. We developed a fluorescence microscopy based assay to examine the Brownian motion of a fluorescent bead conjugated to the genomic dsDNA as it is packaged into the procapsid [11]. We further combined conventional bright-field microscopy with magnetomechanical system for direct visualization of translational motion of a magnetic bead attached to the free end of the viral DNA [24]. Since the motor is too small to be visualized, the micron-sized bead serves as the indicator of the packaging process. These methods can be applied for quantitative kinetic analysis of the DNA packaging process as well as for analyzing the mechanical activities of other viruses. The process is robust, cheap, and straightforward thereby circumventing the need for expensive and specialized instrumentations such as optical and magnetic tweezers [68, 69].

2 Materials

2.1 Specialized Equipment

- Vesicle Prep Pro system (Nanion).
- Mini-Extruder set (Avanti Polar Lipids).
- Axon Axopatch 200B Capacitor Feedback Patch Clamp (Molecular Devices, LLC).
- Axon Digidata 1440A Data Acquisition System (Molecular Devices, LLC).
- PCLAMP 10 CNS software (Clampex and Clampfit) (Molecular Devices, LLC).
- Multimode Atomic Force Microscope (Veeco).
- Ultracentrifuge (Beckman).
- TIRF microscope (Olympus or Nikon).
- Dual-View imager (Photometrics).
- EMCCD Camera (Andor Technology).

2.2 Buffers and Solutions

Prepare all reagents and perform all experiments using Milli-Q water (18.2 MΩ cm⁻¹ resistivity).

- **1× Tris-EDTA (TE) buffer**: 50 mM Tris–HCl (pH 7.8), 10 mM EDTA.
**1x TE/5%glycerol buffer**: 50 mM Tris–HCl (pH 7.8), 10 mM EDTA, 5% glycerol.

**1x Tris-Borate EDTA (TBE) buffer**: 89 mM Tris–HCl, 200 mM boric acid, 2 mM EDTA.

**1x Tris-Acetate EDTA (TAE) buffer**: 40 mM Tris–acetate and 1 mM EDTA.

**1x TMS buffer**: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl2.

**1x TS buffer**: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl.

**PBS buffer**: 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate (pH 7.4).

**Rehydration buffer**: 250 mM sucrose, 1 M NaCl, 5 mM Tris–HCl (pH 7.6).

**BLM Cleaning Solution #1**: Sodium phosphate tribasic (TSP): Place a tablespoon in a 500 mL wash bottle filled with Milli-Q water to prepare a dilute detergent solution.

**BLM Cleaning Solution #2**: 0.1% (vol/vol) HCl.

**Conduction buffers**: 5 mM Tris–HCl (pH 7.9); or 5 mM HEPES (pH 7.9), 1 M NaCl or KCl (typically). Alter the salt concentration depending on desired application.

**Lipid solution A**: DPhPC lipids in n-hexane (concentration: 0.5 mg/mL).

**Lipid solution B**: DPhPC lipids in n-decane (concentration: 20–30 mg/mL).

**Restriction enzyme and DNA ligase reaction buffers** (New England Biolabs).

**Oxygen Scavenger System (OSS) buffer**: 0.5% β-D-glucose, 10 mM β-mercaptoethanol, and GODCAT: 0.2% Glucose Oxidase and 0.25% Catalase.

---

### 3 Methods

#### 3.1 Methods for Single Pore Conductance Measurements

The procedures for cloning, expression, and purification of the reengineered connector proteins have been described previously [40, 70–74]. This protocol focuses on the insertion of the connector channel into lipid membrane to serve as a nanopore and characterize their electrophysiological properties using single channel conduction assays. The connector does not spontaneously insert into the membrane. A two-step process starting with reconstitution into liposomes followed by fusion of liposome–connector complex with planar lipid bilayer is necessary [37].
3.1.1 Prepare Small Unilamellar Liposomes with Membrane-Embedded Reengineered phi29 Connectors

1. Place 1 mL of DPhPC lipids (~1 mg/mL) suspended in chloroform into a small round bottomed flask (see Note 1). Remove the chloroform gently using an automated rotary evaporator or by manual spinning under a slow stream of nitrogen (see Note 2). Place the flask in a vacuum chamber for 1 h to completely remove traces of chloroform.

2. Rehydrate the dried lipid film with 1 mL rehydration buffer containing reengineered connectors (~0.5 mg/mL) and vortex thoroughly.

3. Pass the rehydrated multilamellar proteoliposomes through polycarbonate membrane filters (400 nm) housed inside a mini-extruder set (follow manufacturers’ instructions) to generate homogeneous 400 nm unilamellar liposomes (see Notes 3 and 4).

3.1.2 Set Up Bilayer Lipid Membrane (BLM) Chambers and Instruments for Single Channel Conduction Assays

1. Rinse BLM chambers (Fig. 3a) with ethanol, BLM cleaning solution #1 (containing mild detergent), BLM cleaning solution #2 (to remove residual phosphates), and finally Milli-Q water. Clean Teflon partition (housing a 200 μm aperture) in an eppendorf tube with n-decane, ethanol, and water. Incubate Ag/AgCl electrodes (see Note 5) in bleach for 10 min and then rinse with ethanol and water. Dry all components with an air stream.

2. Affix the Teflon partition on the bottom of cis-chamber using vacuum grease, making sure the aperture is in the center of the chamber. Prepaint the aperture with lipid solution A (<0.5 μL). The Teflon partition separates the chamber into cis-compartment (top) and trans-compartment (bottom). Assemble the chambers and fill them with conduction buffers. Place the head-stage electrode in the trans-compartment and the ground electrode in the cis-compartment. Connect the head-stage to patch clamp current amplifier that is coupled to an analog–digital converter. Place the whole assembly inside a Faraday cage on top of desktop air table to shield the components from electrical and mechanical interferences (Fig. 3a) (see Note 6).

3.1.3 Insert Connectors into Planar Lipid Membrane and Characterize Their Conductance

1. Paint a planar membrane by brushing ~1 μL of lipid solution B directly on the aperture in the Teflon partition (see Note 7).

2. Acquire the data low-pass filtered at a frequency of 1–10 kHz and sampling frequency of 2–200 kHz, as needed (see Note 8).

3. Measure the capacitance \[ C = \frac{I(dV/dt)^{-1}}, \] where \( I \) is the current; \((dV/dt)\) is the applied voltage ramp; and \( C \) is the capacitance] and then calculate the thickness of the lipid membrane \[ d = \frac{(e_0 \times e_s \times A)}{C}, \] where \( d \) is membrane thickness;
$\varepsilon_0$ and $\varepsilon_s$ are permittivity of free space and lipid, respectively; $A$ is the cross-sectional area of the aperture (see Note 9).

4. Add ~1 μL of diluted connector-liposome solution [from Subheading 3.1.1, step 2] into the cis-compartment directly on top of the aperture to fuse with the planar bilayer.

5. Observe discrete stepwise increase in current as connectors insert into the membrane (Fig. 3b). Determine the conductance of the membrane-embedded connectors by two alternative approaches (see Note 10): (a) Apply a ramping potential (e.g., $-100$ mV → $+100$ mV) and determine the slope of the current–voltage trace (Fig. 3c). (b) At constant potential, use the ratio of the measured current jump induced by a discrete step to the applied voltage. Collect data from multiple insertions and plot a histogram to obtain the conductance distribution (Fig. 3d).

---

**Fig. 3** (a) Schematic of Bilayer Lipid Membrane (BLM) chamber for single channel conductance assays. (b) Current trace showing insertion of single connectors into lipid membrane. (c) Current–voltage ($I$–$V$) trace of C-His connectors under ±100 mV. Ramping voltage: 2.2 mV/s. Figures reproduced with permissions from: (a, b) ref. 40 © Macmillan Publishers Ltd.; (c, d) ref. 38 © Royal Society of Chemistry
3.2 Methods for Sensing Single DNA Molecules Using Membrane-Embedded Connectors

The protocol described here is for sensing dsDNA. The same approach can be used for detecting wide range of nucleic acid structures, such as ssDNA, ss- or dsRNA, triplex DNA, quadruplex DNA, and so forth [20, 28, 37, 41, 42].

1. Prepare short dsDNA by chemical synthesis (custom ordered from companies) of two complementary ssDNA fragments followed by annealing. For long kilobase pairs of dsDNA, use plasmid DNA and cut the desired length using restriction enzymes. Purify the DNA by standard polyacrylamide or agarose gel electrophoresis.

2. Add purified DNA samples to the buffer in the BLM chambers.

3. Insert reengineered connectors into planar lipid membrane (see Subheading 3.1).

4. Acquire the data low-pass filtered at a frequency of 1–10 kHz and sampling frequency of 2–200 kHz. Upon channel insertion, a burst of transient current blockage events will be observed, with each blockage event representing the translocation of a single DNA molecule (Fig. 4a, b) (see Note 11).

5. Characterize each of the DNA translocation events using two parameters: (a) Current blockage percentage, represented as \(\frac{(I_{\text{open}} - I_{\text{DNA}})}{I_{\text{open}}} \times 100\), where \(I_{\text{open}}\) is the step size for one-connector insertion, and \(I_{\text{DNA}}\) is the current level during DNA translocation. The channel blockage percentage will be observed to be ~32% (Fig. 4c), which is consistent with the dimensions of the phi29 connector (3.6 nm diameter) and dsDNA (~2 nm in diameter). (b) Dwell time, which is time taken for DNA to pass through the connector. Dwell time distributions typically follow an exponential decay profile (Fig. 4d) (see Note 12).

6. Validate the translocation of DNA by performing Q-PCR [37]. Add DNA on cis-compartment and collect samples from the trans-compartment over a period of time. The number of blockage events from the current trace should closely associate with the number of DNA molecules passing through the pores quantified by Q-PCR (see Note 11).

3.3 Methods for Sensing Single Chemicals or Single Antibodies Using Membrane-Embedded Connectors

For capture and fingerprinting of chemicals and antibodies, it is necessary to perform cloning, expression and mutagenesis of the connector channel. The crystal structure of the connector is known [7, 8], and therefore site-directed mutagenesis can be easily carried out. Various N- and C-termini modifications can be made without affecting the structure and electrophysiological properties of the connector [26, 40, 44].
3.3.1 Capture and Fingerprinting of Single Chemicals

1. For capture of single chemicals, one approach is to use connectors with accessible cysteine residues, which have sulfhydryl side chains suitable for selectively conjugating chemicals or probes. Introduce accessible cysteines by site-directed mutagenesis within the inner wall of the connector, for instance, to generate the K234C mutant [43] (see Note 13). After assembly of the connector, 12 evenly spaced cysteine probes will be present in the same plane of the connector.

---

**Fig. 4** (a) Illustration and (b) current trace showing voltage driven dsDNA translocation through membrane-embedded connectors. 20 bp dsDNA was premixed in both cis- and trans-compartments. Insert in (b): single DNA translocation event with characteristic current amplitude and dwell time. (c) Current blockage percentage and (d) dwell time of DNA translocation events. Red line in (d): Exponential fit. Figures reproduced with permissions from: (a) ref. 26 © American Chemical Society; (b–d) ref. 41 © Elsevier
2. Characterize the conductance behavior of the reengineered cysteine mutant connectors (see Subheading 3.1.3). For reference, K234C channels have a smaller conductance \((2.2 \pm 0.2 \text{ nS})\) \([43]\) and correspondingly smaller cross-sectional area compared to connectors without the cysteine mutation (conductance \((3.2 \pm 0.2 \text{ nS})\) \([37, 38]\)).

3. As proof-of-concept, use chemicals with maleimides or iodoacetamides that form C–S bonds; sulfhydryls that form disulfides under oxidizing conditions; and transthioesterification reactions that link thioesters to cysteines \([75]\). Add the chemicals to the conduction buffer.

4. Monitor the binding of chemicals to the channel wall over time (Fig. 5a). Expect two classes of binding events: transient binding events with a characteristic current blockage amplitude unique to the chemical analyte, but with short dwell time;

---

**Fig. 5** (a) Schematic and (b) current trace showing capture and fingerprinting of single chemicals using cysteine mutant connectors. (c) Differentiation of ethane, thymine, and benzene thioesters with high confidence based on distinctive current blockage percentages. Insert: chemical structures of respective thioesters. Figures reproduced with permissions from ref. 43 © American Chemical Society
and permanent binding events (Fig. 5b, c), indicating covalent bond formation with the cysteine with the same current blockage amplitude as transient events. Count the number of molecules bound from the stepwise blocks in current and deduce the concentration from the number of binding events to a single connector per unit time.

5. Use the current blockage signature to distinguish different chemicals with high sensitivity and specificity (Fig. 5c). Use the dwell time distribution to determine the \( \tau_{on} \) (time between consecutive blockage events), \( \tau_{off} \) (duration of an event), \( k_{on} \) (association rate constant from frequency of association as a function of concentration), \( k_{off} \) (dissociation rate constant from frequency of dissociation as a function of concentration), and finally the \( K_d \) (equilibrium dissociation constant) at the single molecule level (see Note 12).

3.3.2 Capture and Fingerprinting of Single Antibodies

1. For capturing protein antibodies from samples (e.g., patient serum), incorporate capture tags at the terminal ends of the connector. For example, reengineer the connector at the N-terminal with a His-tag (for purification) and at the C-terminal with EpCAM peptide [44] (for capture and fingerprinting of EpCAM antibody in cancer patient serum) (Fig. 6a) (see Note 13). Purify the reengineered connectors to homogeneity [40, 44].

2. Add samples containing EpCAM antibody and a mixture of nonspecific antibodies and proteins.

3. Monitor the binding of specific EpCAM antibodies to EpCAM probe over time based on the current blockage signatures and use the current blockage amplitude (Fig. 6b, c) to distinguish specific from nonspecific events with high confidence (see Note 12). Furthermore, used the transient dwell time events to conduct kinetic studies and determine the \( K_d \) (Fig. 7) [see Subheading 3.3.1, step 5] [44].

3.4 Methods for Imaging Single RNA Nanostructures by Atomic Force Microscopy

Details on RNA nanoparticle constructions have been discussed elsewhere [76]. Mica is most commonly used substrate for imaging RNA nanoparticles due to its atomically smooth surface. Immobilization of RNA nanoparticles on a specially modified substrate is a critical step for AFM imaging. Herein, we focus on chemical modification of mica using silanes that result in a positively charged mica surface that enables adsorption of negatively charged RNA and subsequent imaging by tapping mode in air (Fig. 8).

3.4.1 Preparation of Mica Substrate for Immobilizing RNA Nanoparticles

1. Freshly cleave a sheet of mica to 0.05 mm thickness using razor blades or scotch tape.

2. Mount the mica sheets inside a desiccator.
3. Fill small plastic caps filled with 30 μL APTES (3-aminopropyl-triethoxy-silane) and 10 μL DIPEA (N,N-Di-isopropyl-ethyl-amine), respectively, and place them at the bottom of the desiccator (see Note 14).

4. Put the desiccator under vacuum and fill with argon (see Note 15).

5. Close the desiccator lid and leave for ~2 h to functionalize the mica surface by chemical vapor deposition to generate AP-mica.

6. Remove the reagents and purge the desiccator with argon gas for 5 min.

7. Leave the mica sheets overnight to cure and store under argon atmosphere.

---

**Fig. 6** (a) Schematic and (b) current trace showing specific capture of EpCAM antibodies using membrane-embedded EpCAM peptide probe harboring reengineered connectors. (c) Current blockage distribution showing distinction of EpCAM antibody induced specific binding from nonspecific antibodies in serum. Figures reproduced with permissions from ref. 44 © American Chemical Society
3.4.2 AFM Imaging in Tapping Mode in Air (Fig. 8)

1. Prepare RNA samples (typically 1–20 ng/μL) in TMS buffer (see Note 16).
2. Cut a ~1×1 cm piece of AP-mica and glue it onto an AFM specimen support disk.
3. Place 5–10 μL of RNA sample on AP-mica and incubate for 2–3 min.
4. Flush the sample thoroughly with 2 mL of HPLC grade water (see Note 17) and leave the samples to dry overnight under argon atmosphere.

Fig. 7  (a) Current trace showing transient current blockage events induced by EpCAM antibody binding to EpCAM peptide probe for kinetic studies of peptide probe–antibody interactions. \( \tau_{\text{on}} \) (time between consecutive events) and \( \tau_{\text{off}} \) (dwell time) are shown.  (b, c) Event distribution of \( \tau_{\text{on}} \) and \( \tau_{\text{off}} \), respectively. (d, e) Frequency of association and dissociation as a function of antibody concentration to yield association rate \( (k_{\text{on}}) \) and dissociation rate \( (k_{\text{off}}) \), respectively to generate equilibrium dissociation constant \( (K_d) \). Figures reproduced with permissions from ref. 44 © American Chemical Society
5. Mount the samples on the AFM stage, load the tip, and align the photodiodes following manufacturer’s instructions (see Note 18).

6. Find the resonance frequency of the AFM probe and engage the surface (see Note 19).

7. Adjust the set-point voltage slowly until the surface is visible, optimize the feedback gains (as needed) and initiate imaging of the surface.

3.5 Methods for Determining the Stoichiometry of RNA on phi29 Motor by Single Molecule Photobleaching Assay

Methods for fluorescent labeling of pRNA during in vitro transcription using Cy3-AMP and isolation of procapsid/Cy3-pRNA complex by sucrose gradient sedimentation have been described previously [11, 77, 78]. Verify that the Cy3-labeled pRNA is competent in procapsid binding and DNA packaging by in vitro virion assembly [79]. Herein, we focus on the experimental setup for single molecule photobleaching assay using total internal reflection single-molecule fluorescence imaging system with a top prism (Fig. 9a, b).
1. Prepare a sample chamber using a quartz slide and a glass coverslip, with double-sided tape as spacer. Make sure the chamber has an inlet and an outlet for solution exchange.

2. Infuse 0.1 mg/mL anti-procapsid IgG (Proteintech Group Inc.) solution in the chamber and incubate for 2 h at room temperature. Wash the chamber with 3 chamber volumes of 1× TMS buffer.

3. Dilute the procapsid/Cy3-pRNA complex in TMS buffer to desired concentration (see Note 20). Infuse the solution to the

---

**Fig. 9** Single-molecule photobleaching for direct counting of RNA molecules. (a) Illustration of single molecule TIRF setup with simultaneous dual-color detection capabilities. (b) Experimental design for direct counting of pRNA molecules in phi29 DNA packaging motor. (c) Typical TIRF image of single phi29 motors containing Cy3 labeled pRNA. (d) Typical fluorescence photobleaching trace showing six stepwise drop of fluorescence intensity over time. (e) Typical histogram showing distribution of photobleaching steps comparing experimental results with prediction based on 70% Cy3 labeling efficiency. Figures reproduced with permissions from: (a, b, d, e) ref. 11 © EMBO Press
IgG coated chamber and incubate at room temperature for 30 min. Wash with 3 chamber volumes of 1× TMS for three times. Add OSS buffer before fluorescence imaging.

4. Place the chamber on the TIRF microscope. Use 60× oil immersion objective for imaging. Place a drop of 100% glycerol on top of the quartz slide and then position the quartz prism on top of the slide. Use 532 nm laser for excitation of Cy3. Fine tune the position of the prism to adjust the location of the total internally reflected beam right above the field of view in the microscope. Conduct live imaging of the sample to check whether the density of the fluorescent spots is appropriate for detection of single procapsid/pRNA complex.

5. Move to a fresh region in the chamber and adjust the laser power to photobleach fluorophores at a reasonable speed so that the photobleaching steps can be clearly distinguished (see Note 21).

6. Acquire sequential fluorescence images with an exposure time of 200–400 ms continuously (Fig. 9c) (see Note 21).

7. Analyze the acquired images by computer program (for example Kinetic imaging by Andor Technologies) to generate the plot of fluorescence intensity vs. time for each spot (Fig. 9d, e) (see Note 22).

3.6 Methods for Single Molecule Distance Measurement of RNA by FRET

For distance measurements using single molecule FRET, it is important to have single fluorophores labeled at the desired locations on the RNA molecules. In addition, functional moiety, such as biotin, can be used for immobilization of the single RNA molecule onto the imaging surface. The labeling procedures for RNA molecule through transcription, chemical conjugation, or customized oligo synthesis have been reported previously [11, 76, 80, 81]. This protocol focuses on FRET detection using single molecule TIRF and structural analysis of RNA in presence or absence of Mg2+ using dual-labeled pRNA-3WJ as an example (Fig. 10a) [12].

1. Mix the individual biotin-3WJ-a, Cy3-3WJ-b and Cy5-3WJ-c strands at equal molar concentration in water. Dilute the assembled Cy3/Cy5-3WJ complex to 33 pM with TS buffer for studying in the absence of Mg2+, or with TMS buffer for studying in the presence of Mg2+.

2. Incubate the sample chamber with 1 mg/mL biotin-BSA (Sigma) for 15 min. Wash the chamber with PBS buffer three times.

3. Infuse 0.33 mg/mL streptavidin (Prozyme) solution into the chamber and incubate for 15 min. Wash the chamber three times with TS buffer or TMS buffer.
4. Incubate the diluted Cy3/Cy5-3WJ sample in the streptavidin coated chamber for 15 min. Prepare OSS buffer using TS or TMS and add the corresponding OSS into the chamber right before TIRF imaging.

5. Follow step 4 in Subheading 3.5 for TIRF imaging of the sample. Use 532 nm laser as excitation light source. Place Cy3/Cy5 filter set in the Dual-View imager, which is aligned in the light path for fluorescence signal collection. Collect both Cy3 and Cy5 signals simultaneously with EMCCD camera (Andor Ixon 887V). Acquire sequential images with an exposure time of 300 ms continuously for 2000 frames.

6. Analyze the sequential images by computer program (for example Kinetic Imaging by Andor Technologies). Use Field Split function in Kinetic Imaging to overlay Cy3 and Cy5 signals. Assign pseudo color green for Cy3 and red for Cy5. Identify fluorescent spots that display yellow color, which is an
indication of colocalization of Cy3 and Cy5. Plot the fluorescence intensity of these overlapped spots versus time to generate FRET traces (Fig. 10b).

7. Analyze each FRET traces and calculate the FRET efficiency based on the following equation (see Note 23):

\[ E = \frac{\Delta I_D}{I_{D0}} = \frac{(I_D - I_{DA})}{(I_D - I_{0Cy3})} \]  

(1)

8. Convert FRET efficiency to distance based on the following equation (see Note 24):

\[ R = R_0 (1/E - 1)^{1/6} \]  

(2)

9. Summarize FRET efficiency and calculated distances into histograms and compare the results for the sample with and without Mg\(^{2+}\) (Fig. 10c).

### 3.7 Methods for Observing DNA Packaging by Optical Fluorescence Microscopy

To observe DNA translocation, it is necessary to anchor the phi29 procapsid on a glass slide and attach fluorescent beads to the genomic DNA by biotin-streptavidin interactions (Fig. 11a). The motor packaging can be stalled using nonhydrolyzable ATP (\(\gamma\)-s-ATP) and restarted simply by adding ATP. The motion process can then be studied by fluorescence imaging [11].

#### 3.7.1 Generate Biotinylated phi29 Genomic DNA for Labeling with Fluorescent Bead

1. Mix 150 μL phi29 genomic DNA-gp3 (19.3 kb) with 2 μL EcoRI and 2 μL HaeIII restriction enzymes in 1× reaction buffer. Bring the final volume to 200 μL using 1× TE/5% glycerol. Incubate the reaction at 37 °C for 4 h.

2. Analyze the DNA by agarose gel electrophoresis (0.5% agarose gel containing 15% glycerol) and purify the band of interest (9.7 kb).

3. Add NaCl at a final concentration of 0.5 M and PEG 8000 at a final concentration of 10% to the eluted DNA and mix well. Leave the mixture on ice for at least 2 h and then centrifuge at 16,000× rcf for 30 min at 4 °C. Remove the supernatant and resuspend the pelleted DNA in 50 μL 1× TE/5% glycerol.

4. Prepare biotinylated linker DNA by annealing of DNA oligo 1 (example sequence: 5’-/5Phos/AAT TGT AAC ATG TTC GGA TCC ACG AAA AAA AAA AAA AAA AAA AA-3’) and DNA oligo 2 (example sequence: 5’-CGT GGA TCC GAA CAT G/iBiodT/TAC-3’) at equal molar concentration.

5. Mix 40 μL the purified EcoRI fragment from step 5 with 15 μL biotinylated linker DNA from step 6 and 2 μL T4 DNA ligase in 1× T4 DNA ligase buffer. Incubate the mixture at 16 °C for 16 h.
6. Precipitate the ligated DNA following step 3. Resuspend the DNA pellet with 50 μL 1× TE/5% glycerol. Store the DNA at −80 °C until use.

3.7.2 Preparation of Stalled Packaging Intermediate

1. Take 1 μL streptavidin-coated microspheres (Dragon Green, 0.53 μm) (Bang laboratories, Inc) and add 10 μL TMS. Mix well and spin at 16,000× rcf for 5 min. Remove supernatant and resuspend the beads in 10 μL TMS. Repeat this wash step three times.

2. Sonicate the streptavidin-coated beads in 20 μL 1× TMS in a Branson sonicator for 30 min. Add 2 μL 10% BSA and 1.5 μL biotin-EcoRI DNA from Subheading 3.7.1 step 6 to the bead solution. Incubate on a tumbler for 1 h at room temperature.

3. Centrifuge the mixture at 16,000× rcf for 5 min and resuspend the bead–DNA in 6 μL TMS.

4. Mix the bead-labeled DNA with 10 μL phi29 procapsid, 100 ng pRNA, 3 μL of 10 mM ATP containing 50 nM γ-s-ATP, and 6 μL of 0.6 μM gp16. Incubate at room temperature for 1 h (see Note 25).

5. Dilute the mixture with TMS buffer and flush into the imaging chamber coated with anti-procapsid IgG. Incubate at room temperature for 30 min. Wash the chamber with TMS buffer for three times.

3.7.3 Real-Time Observation of DNA Translocation with Fluorescence Microscopy

1. Place the imaging chamber on the fluorescence microscope. Image with a 60× oil immersion objective. Use FITC filter cube set for imaging of fluorescein labeled beads. Look for tethered beads that are still mobile in solution. Set the exposure time for 30 ms.

2. Flush in the chamber with 1× TMS containing 1 mM ATP and 50 nM γ-s-ATP.

3. Record a movie (1 frame/s for 600 s) right after the addition of the buffer to track the beads.

4. Flush in the chamber with TMS containing 1 mM ATP and 0.12 μM gp16, which will restart the stalled DNA packaging intermediate.

5. Record a movie (1 frame/s) right after the addition of the buffer until the beads stop the motion (see Note 26).

6. Analyze the movies to track the motion of the beads (Fig. 11b) (for example using Tracker software from Andor Technologies or SPOTTRACKER [82]).
3.8 Methods for Observing DNA Packaging by Combining Optical Microscopy and Magnetomechanics

3.8.1 Preparation of Stalled Packaging Intermediate Labeled with Magnetic Beads

1. Cut a small piece of glass slide with a dimension about \(5 \times 5 \times 0.22\) mm\(^3\) and coat the glass surface with anti-procapsid IgG by dipping the glass piece in a solution of 0.1 mg/mL anti-procapsid IgG in PBS for overnight at 4 °C.

2. Place the IgG coated glass piece into the sample chamber. The edge of the IgG coated glass serves as the glass wall for immobilization of the motor intermediates (Fig. 11c).

3. Mix 3 \(\mu\)L gp3-DNA with 10 \(\mu\)L phi29 procapsid, 100 ng pRNA, 3 \(\mu\)L 10 mM ATP containing 50 nM \(\gamma\)-s-ATP, and 6 \(\mu\)L 0.6 \(\mu\)M gp16. Incubate at room temperature for 1 h.

4. Infuse the packaging mixture into the sample chamber and incubate for 30 min to allow the intermediate attach to the anti-procapsid IgG coated glass surface, and then wash with TMS buffer three times to remove the unbound packaging intermediate and free gp3-DNA.

5. Conjugate anti-gp3 IgG with NHS-activated magnetic beads following the coupling protocol provided by manufacture.

Fig. 11 Direct observation of phi29 DNA packaging. (a) Experimental design for observation of DNA packaging using fluorescence microscopy. (b) Top: Sequential images showing the motion of the fluorescein labeled microsphere that is attached to the DNA. Bottom: Plot demonstrating changes of the moving range of the tethered microsphere over time during DNA packaging process. (c) Experimental design for observing DNA packaging using bright field microscopy combined with magnetomechanics. (d) Plot of tethered DNA length versus time after the packaging intermediate is restarted. (e) Plot of DNA packaging rate calculated from (d) versus percentage of packaged DNA. Figures reproduced with permission from: (a, b) ref. 11 © EMBO Press; (c–e) ref. 24 © American Institute of Physics
6. Add the IgG coated magnetic beads into the sample chamber and incubate for 30 min to allow for binding to the open ends of prepackaged gp3-DNAs.

1. Place the sample chamber on the stage of the microscope.

2. Fix a permanent magnet onto a three dimensional translation stage (Thorlabs LT3) and place the magnet beside the microscope. Set the magnet to the same height as the microscope stage. Tune the position of the magnet to achieve desired magnetic force that is exerted on the magnetic bead and maintain at a constant force (see Note 27).

3. Image with a 50× objective under bright field condition. Look for tethered beads that are still mobile in solution.

4. Flush in the chamber with 1× TMS containing 1 mM ATP and 0.12 μM gp16, which will restart the stalled DNA packaging intermediate (see Note 26).

5. Record a movie right after the addition of the buffer until the beads stop the motion.

6. Analyze the movies to track the motion of the beads (Fig. 11d, e) (for example using Tracker software from Andor Technologies or SPOTTRACKER [82]). Measure the length of tethered DNA during packaging and plot it over time to obtain information on DNA packaging kinetics.

4 Notes

1. DPhPC is a zwitterionic lipid. Other lipids may also be used.

2. The formation of a uniform thin lipid film is critical for forming high quality liposomes.

3. Store the proteoliposomes at −20 °C for long term storage and 4 °C for short term storage.

4. If necessary, perform a 5–20% (wt/vol) linear sucrose gradient sedimentation to separate the proteoliposomes from free connectors and naked liposomes.

5. Do not store electrodes in contact with metals and avoid exposure to light.

6. If necessary, use salt bridges to facilitate better electrical coupling of electrodes with the amplifier. If needed, setup a perfusion system for exchange of buffers, and a bilayer thermocycler for temperature control. Make sure that all the instrument components are connected to a common ground, and the ground connection is isolated from all circuits.
7. Gently remove excess lipid from the aperture using air bubbles generated from the pipette tip. Wait for a few minutes for the membrane to stabilize.

8. To avoid sampling artifacts, set the sampling frequency to be at least 5 times higher than the filter cutoff frequency.

9. The optimal thickness of lipid bilayer is ~5 nm. If the membrane is too thin, the connector will be unstable; if the membrane is too thick, proteoliposomes will not fuse.

10. Determine the experimental conditions (e.g., buffer, voltage) where the connector channel remains stable without any voltage gating properties.

11. Phi29 connector exercises a one-way traffic property for dsDNA translocation from N-terminal to C-terminal end. Vesicle fusion of proteoliposomes with a planar bilayer can result in the connector oriented with either the N-terminal or the C-terminal facing the cis-compartment. If no translocations are observed, switch the polarity, and DNA translocation will occur in the other direction, as we have shown previously [26].

12. Acquire a large number of translocation events to obtain statistical significance.

13. For phi29 connectors, addition and deletion of 25 amino acids at the terminal ends has no effect on the formation and stability of the channel. Nevertheless, thoroughly characterize to discover any adverse effects arising from the mutations.

14. Use only fresh APTES stored under argon. If mica displays surface heterogeneities after functionalization, most likely, the APTES has polymerized. In that case, vacuum distill APTES under argon atmosphere.

15. Use an overflow gauge to ensure that pressure does not build up inside the desiccator after sealing the desiccator with vacuum grease.

16. The surface density of RNA nanoparticles will vary based on substrate functionalization, concentration, size and shape of the samples. Optimize the concentration of RNA samples to ensure minimal overlap of adsorbed structures.

17. Use high quality water to eliminate any nanometer-sized particulates that will accumulate on the substrate and interfere with imaging of the samples.

18. Depending on the size of RNA nanoparticles, ultrasharp silicon tips (1 nm; spring constant of 40 N/m; resonance frequency of >300 kHz) may be needed for acquiring high resolution images.

19. Ultrasharp tips are very fragile and expensive. Exercise great care when approaching the surface using only low forces.
20. Optimize the concentration to observe discrete fluorescent spots in the field of view.

21. Adjust the laser power and exposure time to obtain adequate signal-to-noise ratio for single fluorophores.

22. Acquire a large number of photobleaching traces to obtain statistical significance.

23. FRET efficiency can be affected by the orientation of the fluorophores placed in the molecules. Cautions should be taken when designing the fluorescence labels on the RNA molecules to ensure structural flexibility for free rotating of the fluorophores, thus reducing the orientation effect in FRET efficiency determination.

24. Additional distance generated from the size of the fluorophore itself should be subtracted when measuring the distance between two nucleotides based on the FRET results.

25. Optimal concentration of $\gamma$-s-ATP needs to be titrated by in vitro virion assembly assay for different batches of $\gamma$-s-ATP.

26. The stalled motor may not resume packaging immediately upon the addition of the restart solution. Such a delay could be a few minutes and varies from case to case.

27. Maintain the magnetic force around 1 pN so that the gp3-DNA will not be overstretched, or the motor will not be stalled due to high force. Calibration of magnetic force applied on the spherical magnetic beads has been described previously [24].

Acknowledgments

The research was supported by NIH grants U01-CA151648 and R01-EB012135. Funding to P.G.’s Endowed Chair in Nanobiotechnology position is by the William Fairish Endowment Fund. P.G. is a cofounder of Biomotor and Nucleic Acid Nanotechnology Development Corp., Ltd.

References


DNA against a large internal force. Nature 413:748–752


48. Khisamutdinov EF, Jasinski DL, Guo P (2014) RNA as a boiling-resistant anionic polymer material to build robust structures with defined shape and stoichiometry. ACS Nano 8:4771–4781
58. Sengupta P, Jovanovic-Talisman T, Lippincott-Schwartz J (2013) Quantifying spatial organization in point-localization superresolution...
images using pair correlation analysis. Nat Protoc 8:345–354
64. Forster T (1946) Energiewanderung und fluoreszenz. Naturwissenschaften 6:166–175