

Viral DNA Packaging Studied by Fluorescence Correlation Spectroscopy

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ABSTRACT The DNA packaging machinery of bacteriophage T4 was studied in vitro using fluorescence correlation spectroscopy. The ATP-dependent translocation kinetics of labeled DNA from the bulk solution, to the phage interior, was measured by monitoring the accompanied decrease in DNA diffusibility. It was found that multiple short DNA fragments (100 basepairs) can be sequentially packaged by an individual phage prohead. Fluorescence resonance energy transfer between green fluorescent protein donors within the phage interior and acceptor-labeled DNA was used to confirm DNA packaging. Without ATP, no packaging was observed, and there was no evidence of substrate association with the prohead.

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Viral DNA packaging machines, “packasomes”, are interesting biophysical subjects because they utilize the strongest motors identified to date (1). For bacteriophage T4, the minimal in vitro packasome includes gene products (gp) 17 (the terminase and DNA translocating ATPase) and 20 (the dodecameric entrance portal of the preformed empty capsid prohead). DNA packaging can be measured by a DNase protection assay (2): nuclease is used to selectively digest free DNA in solution, but not DNA inside proheads, and the products are size-analyzed on a gel. This assay has been used to investigate the packasome DNA translocation kinetics, identify substrate requirements, measure packaging efficiency, and examine the inhibition of packaging by non-hydrolyzable ATP analogs (2).

In this Letter, we discuss the novel application of fluorescence correlation spectroscopy (FCS) to monitor the kinetics of DNA packaging in near real-time. FCS measurements are noninvasive, an advantage over the DNase protection assay. The principle behind the FCS assay is to monitor the change in the apparent diffusion coefficient when free (labeled) DNA is translocated into a prohead. For an in vitro T4 system, double-stranded DNAs ranging from 20 basepairs (bp) up to the full length 170 kbp genome can be packaged (2,3). The ability to measure DNA packaging using FCS relies on a large difference in the diffusion coefficients of the prohead and substrate. The diffusion coefficient of the prohead is calculated to be $4.4 \mu\text{m}^2/\text{s}$ (4); therefore, we chose to use a 100 bp substrate with a calculated diffusion coefficient of $38.0 \mu\text{m}^2/\text{s}$ (5). In our experimental setup a $40\times 1.2 \text{ N.A.}$ water immersion objective is used to focus 514.5 nm laser light ($2\text{--}10 \mu\text{W}$) to a diffraction-limited volume. The volume is imaged onto an avalanche photodiode through a $50 \mu\text{m}$ pinhole that rejects out-of-focus light and restricts the fluorescence emission to a subfemtoliter volume. The autocorrelation function for n fluorescent species traversing a

3D Gaussian volume with radius ω_0 and half-axial height z_0 is:

$$G(\tau) = \frac{1}{N} \sum_{i=1}^n f_i [1 + 4D_i\tau/\omega_0^2]^{-1} [1 + 4D_i\tau/z_0^2]^{-1/2},$$

where τ is the lag time, N is the number of molecules in the volume, and f_i are the fractions of the corresponding diffusion coefficients D_i (6). To demonstrate the robustness of FCS to obtain diffusion coefficients and concentrations, we determined $G(\tau)$ for various concentrations of rhodamine (R6G) solutions. Fig. 1 A is a plot of the global fits of the FCS data using a single-species biophysical model. The extrapolated $G(0)$ values are used to determine N , which scales linearly with R6G concentration (Fig. 1 A, inset). DNAs with a single 5'-R6G fluorophore were used as the substrates for the packasomes. Our in vitro packaging system requires the use of high molecular weight PEG 20,000 and polyanions that increase the solution viscosity and can interact with the DNA (2), resulting in decreased diffusibility. Fig. 1 B shows a comparison of the FCS curves for the 100 bp DNA in Tris buffer and in packaging buffer. A single diffusion constant of this substrate was measured to be $38.1 \mu\text{m}^2/\text{s}$ in Tris buffer, which is in accord with the expected value. The correlation curve for the same DNA in the reaction buffer exhibited longer time decays indicating reduced diffusibility (Fig. 1 B). Additionally, a single-species model does not precisely fit the data, which indicates the heterogeneity of the environment. It may be the case that the DNA interacts with the polymers and/or the volume excluding effect of PEG hinders free DNA diffusion. However, we

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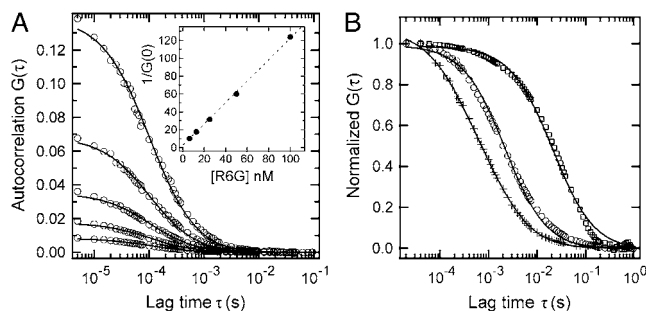


FIGURE 1 (A) FCS of varying concentrations of R6G. The solid lines are fits based on a single diffusing species with $D = 280 \mu\text{m}^2/\text{s}$. The inset plots the inverse $G(0)$ values as a function of R6G concentration. The dotted line is a linear fit to the data and has a slope of 1.2 molecules/nM. (B) FCS of R6G-labeled 100 bp DNA in 10 mM Tris buffer (crosses), packaging buffer (circles), and labeled proheads in reaction buffer (squares). The solid lines are fits using a single-species model.

estimated an average diffusion coefficient of $11.7 \mu\text{m}^2/\text{s}$ from a single-species fit. The diffusion coefficient of the prohead was measured to be $0.66 \mu\text{m}^2/\text{s}$ (Fig. 1 B), using the products from an overnight packaging reaction, in which case $\sim 100\%$ of the R6G-labeled DNA is translocated to the proheads' interior. The diffusion coefficient for the prohead in reaction buffer is also slower than the calculated diffusion in water.

The kinetics of DNA packaging were measured by taking FCS measurements at 20 s intervals for 30 min. The reaction mixture contained: proheads-gp20, gp17, and ~ 5 times molar excess of 100 bp DNA over the proheads. DNA packaging was initiated by the addition of ATP. The result of the FCS time course is shown in Fig. 2 A. The most striking feature about the FCS curves is the gradual increase of $G(0)$ as the reaction proceeds. The time dependence of $G(0)$ is due to the apparent decrease in fluorophore concentration as a result of multiple DNAs being packaged per prohead. Because the prohead is essentially a point particle, multiple packaged substrates appear as a single diffusing entity. Therefore, FCS was used to directly establish that short DNA fragments—a fraction of the T4 genome in size—can be

sequentially packaged in vitro, in agreement with previous observations (2,7). We estimate a 22% decrease in the apparent substrate concentration from the change in inverse $G(0)$ values, which indicates an average of 4–5 100 bp fragments per prohead. This analysis assumes that the reaction proceeds in a homogeneous manner. If, however, there is a heterogeneous distribution in the number of DNAs per prohead (brightness), then the amplitude of the FCS curves will be weighted by the square of the brightness of each species (6). For example, if there are two species in the volume and one is five times brighter than the other, the apparent volume occupancy will be 1.4, not 2.0. Thus, the concentration of free labeled DNA will be underestimated as the proheads' brightness increases. In an effort to minimize heterogeneity, the DNA/prohead ratio was ~ 5 , in which case the available DNA is far less than amount required for headful packaging (~ 1700 DNAs/prohead).

We estimated the kinetics of DNA packaging by global analysis of the FCS curves with a two-species, homogeneous brightness biophysical model. The diffusion coefficients, $D_1 = 11.7 \mu\text{m}^2/\text{s}$ for the free 100 bp DNA and $D_2 = 0.66 \mu\text{m}^2/\text{s}$ for the prohead were fixed during fitting, and the corresponding fractions $f_1 + f_2$ were free parameters constrained to unity. The fraction of free DNA (f_1) versus time is plotted in Fig. 2 B. The decrease in free DNA concentration over time fits very well to a single exponential decay, implying a first-order process: $d[\text{DNA}_{\text{free}}]/dt = -k[\text{DNA}_{\text{free}}]$, with $k = 0.106/\text{s}$. The packaging kinetics were also analyzed from the change in the volume occupancy, N , over time (Fig. 2 C). The apparent decrease in fluorophore concentration due to DNA packaging was also found to fit well to a first-order process, $dN/dt = -kN$, with $k = 0.109/\text{s}$. This volume occupancy analysis is independent from the diffusion coefficient analysis, and the agreement between the rate constants deduced from both models supports the robustness of FCS to measure DNA packaging kinetics. For a negative control, ATP was omitted from the reaction. As shown in Fig. 2 D, the FCS curves were similar during the 30 min reaction, which indicates no DNA packaging. Moreover, when the two-species model was used for global fits to the FCS curves, the average amplitude of the free DNA was 96% (Fig. 2 E).

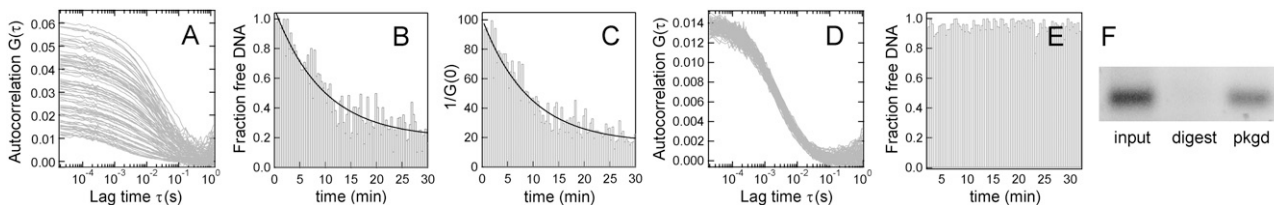


FIGURE 2 DNA packaging kinetics. (A) FCS acquired at 20 s intervals for the 30 min reaction with ATP. In general, the $G(\tau)$ amplitude increases over time. Kinetic profiles based on the diffusion coefficient fractions (B), and volume occupancy number (C). The solid lines are single exponential fits to the data. FCS for a 30 min reaction without ATP (D), and the corresponding kinetic profile based on the diffusion coefficient fractions (E). (F) Gel analysis of the DNase protection assay. The first lane is the total input DNA of the reaction (no DNase); the second lane is the DNase positive control (reactions lacking proheads and ATP); and the last lane is the packaged (*pkgd*) DNA protected from digestion.

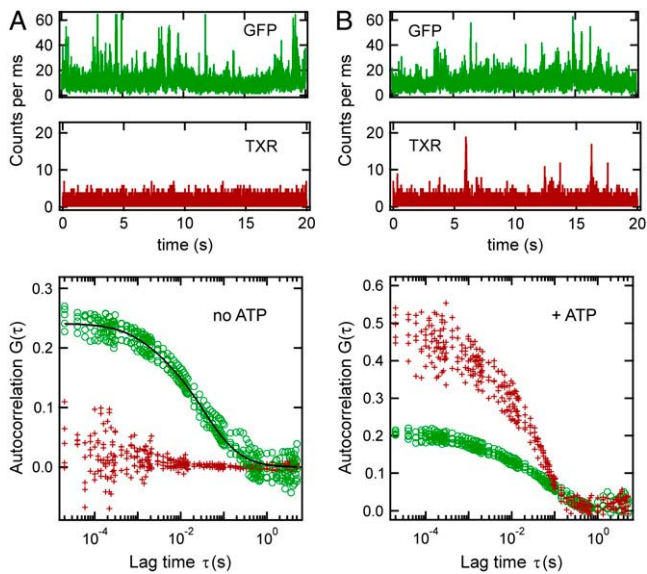


FIGURE 3 FRET-FCS analysis of overnight packaging reactions without ATP (A) and with ATP (B). Intensity time traces of the prohead-internalized donor (GFP, green), and acceptor (TXR, red) labeled DNA, are shown in the top panels for a 20 s FCS run. Traces were binned to 1 ms. The bottom graphs are overlaid FRET-FCS plots for 5 separate FCS runs (GFP, circles; TXR, crosses).

Therefore, our data suggest minimal association of the DNA with the prohead. If the DNA substrate and prohead associate in the absence of ATP, we would expect to see a change in DNA diffusion, but not a difference in $G(0)$. The DNase protection assay confirmed the FCS results: 70–80% of the input DNA was protected in the 30 min reaction (Fig. 2 F).

A fluorescence resonance energy transfer (FRET) FCS assay was also used to confirm that DNA was translocated inside the prohead interior, and rule out any nonspecific associations that could also yield a change in DNA diffusibility. The FRET donor/acceptor pair used was green fluorescent protein (GFP)/Texas Red (TXR). The T4 capsid targeting peptide sequence was added to GFP to localize the molecules to the prohead interior during *in vivo* capsid assembly; each prohead contained ~ 100 GFPs (8). TXR-labeled 100 bp DNAs were used as the substrates, and reactions were incubated overnight to maximize DNA packaging. FCS was performed using 488 nm laser light to excite the donor, and a dichroic mirror was used to split the GFP and any TXR emissions onto separate detectors. Fig. 3 A shows the FRET-FCS data for DNA packaging in the absence of ATP. The average GFP-prohead FCS profiles displayed two diffusion coefficients (amplitudes): $D_1 = 0.66 \mu\text{m}^2/\text{s}$ (0.72), similar to that obtained using proheads filled with R6G-labeled 100 bp DNA, and a much faster $D_2 = 12.0 \mu\text{m}^2/\text{s}$ (0.27). We believe that the faster diffusion coefficient is due to free GFPs

released from proheads that were damaged during handling. The flat FCS curve for the TXR channel is due to the fluorophore's low quantum yield with 488 nm excitation, and the uncorrelated signal is primarily from the random background counts. When ATP is added to the packaging reaction, the TXR emissive rate is increased, as can be noted by the non-zero FCS curve (Fig. 3 B). This increase in TXR fluorescence is due to FRET, which can only occur if the donor and acceptor are within ~ 8 nm, and is further evidence of labeled DNA being translocated to the prohead interior. A robust diffusion analysis based on the acceptor autocorrelation was not attempted because of the heterogeneity of this FRET system, which is evident from the variations in the acceptor FCS curves (Fig. 3 B). This heterogeneity is most likely due to fluctuating donor-acceptor distances due to diffusion of the donors and acceptors inside the proheads. Nonetheless, the FRET-FCS experiments confirm that the DNAs are specifically translocated from the bulk solution to the proheads' interior in an ATP-dependent manner, and these results support the kinetic packaging data.

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