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Measurement of DNA Polymerase Incorporation Kinetics of Dye-Labeled Nucleotides Using Total Internal Reflection Fluorescence Microscopy

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Supporting Information

ABSTRACT: We report a method for the rapid and automated measurements of the incorporation kinetics of fluorescent dye-labeled nucleotides by DNA polymerases without using stopped-flow and quench-flow methods. Total internal reflection fluorescence microscopy is used to monitor the incorporation of fluorescently labeled nucleotides by DNA polymerase into surface-bound primed DNA templates, and a microfluidic system is used to perform the reactions. We successfully demonstrated the method using Bst DNA polymerase and a set of coumarin-labeled nucleotides. Our method allows the rapid acquisition of polymerase kinetics for implementing and improving DNA sequencing technologies that rely on labeled nucleotides and DNA polymerases.

The remarkable fidelities of DNA polymerases are determined by the binding and incorporation kinetics of the nucleotide substrates.[1−3] The pre-steady-state equilibrium dissociation and incorporation rate constants are usually determined by stopped-flow methods in combination with fluorescence measurement and quench-flow methods followed by gel electrophoresis analysis or radiometric assays.[4−8] Fluorescently labeled DNA polymerases and fluorescent nucleotide analogues can also be used to characterize conformational dynamics and reaction kinetics by Förster resonance energy transfer (FRET) imaging.[9−12] We report a method for the rapid and automated measurement of the incorporation kinetics of fluorescent dye-labeled nucleotides by DNA polymerases. We use total internal reflection fluorescence microscopy (TIRF) coupled to a custom-designed flow cell with fluidic and temperature control for real-time fluorescence imaging of the enzymatic reactions on the surface. The basic setup is illustrated in Figure 1 (more detail in Figure S1 of the Supporting Information). The shallow penetration depth of the evanescent wave created by total internal reflection (TIR) allows for the selective excitation of only the molecules very close to the surface (~160 nm in our case),[13] allowing the real-time monitoring of the incorporation of the labeled nucleotides into primed DNA templates immobilized on the surface of a glass substrate.

Glass coverslips are processed,[14] functionalized with poly-$(dT)_{50}$ (detail in the Supporting Information), then assembled onto a flow cell consisting of nine 40 mm × 2 mm × 100 μm channels cut from double-side adhesive silicone tape, and attached to an anodized aluminum or stainless steel plate with 1 mm holes drilled for fluidic connections. The modular flow cell is mounted into an aluminum encasement block of a temperature control unit comprised of thermoelectric modules sandwiched between the block and a liquid-cooled aluminum heatsink, which is designed to mount on a motorized XY microscope stage. A thermistor is fixed to the encasement block to provide feedback to a temperature controller. Fluid control is managed by a syringe pump connected to a nine-port rotary valve. All components are controlled through a computer using custom software written in C++ to automate the entire process, including temperature control, delivery of solutions for multistep reactions, and real-time fluorescence imaging.

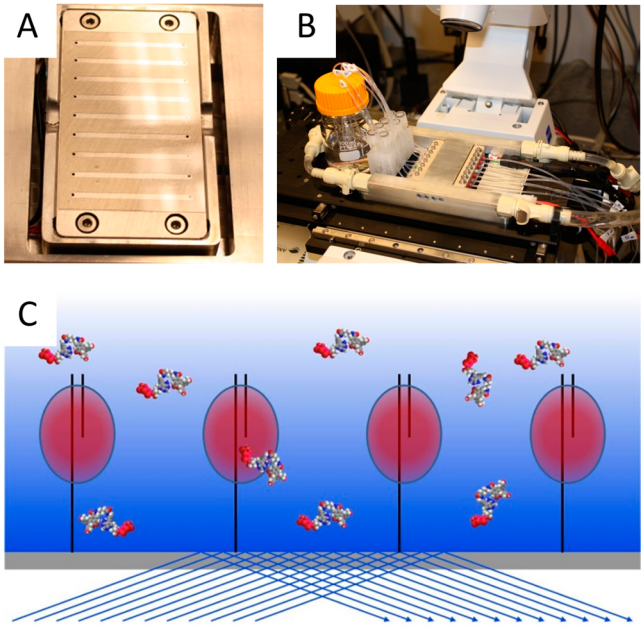

Figure 1. Fluidic system and TIRF for rapid measurement of incorporation kinetics of dye-labeled nucleotides by surface-bound DNA polymerases. (A) Flow cell with nine fluidic channels. (B) Flow cell with a fluidic interface mounted to a temperature control unit on a microscope stage. (C) Schematic of the nucleotide incorporation reaction on surface-bound DNA polymerases with TIR illumination.

To prepare the fluidic channels for the reactions, a solution of 0.5 μM DNA template with a poly(dA) tail and a specific

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primer (1 μM) designed to incorporate a particular base (Table S1 of the Supporting Information) in 2× saline sodium citrate buffer with 0.02% Triton X-100 (2× SSCt) was passed into each separate channel. The DNA templates were hybridized to poly(dT)$_{50}$ on the surface and the primers by first increasing the temperature to 65 °C and holding at that temperature for 5 min and then decreasing the temperature to 25 °C at a rate of 0.1 °C/s. The channels were then washed with 2× SSCt, followed by 1× Bst buffer [20 mM Tris, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, and 0.1% Triton-X 100 (pH 8.8)] to prepare for the polymerase reaction. To ensure that most primers were bound with a polymerase prior to the incorporation reaction, Bst DNA polymerase large fragment at 1 unit/μL (0.13 μM) in a 1× Bst buffer solution containing 0.1 mg/mL bovine serum albumin (BSA) and 0.019 mg/mL *Escherichia coli* single-stranded binding protein (SSB) was passed into and incubated in the channels for at least 5 min.

The reactions were conducted with a series of nucleotide concentrations at 25 °C. Each solution contained 0.2 unit/μL (0.025 μM) Bst polymerase, 8.5 μg/mL SSB, 0.1 mg/mL BSA, and the desired concentration of the custom-synthesized coumarin-labeled nucleotide (see Figure S2 of the Supporting Information). The solution was injected into the channel at a linear flow rate of 100 mm/s, and time-lapse TIRF images were immediately acquired. The objective-based TIRF system consisted of an inverted fluorescence microscope and a TIRF slider (AxioObserver Z1 and TIRF 3 Slider, Carl Zeiss). A 100× oil objective lens with a NA of 1.46 (Alpha Plan Apochromat, Carl Zeiss) was used for both laser excitation and fluorescence detection. A 405 nm diode laser coupled to the slider via a polarization-preserving single-mode broadband optical fiber was used for excitation. The excitation power was 19.5 mW after the objective, and the illumination angle was adjusted slightly beyond the TIR angle using a piezo-driven mechanism. The fluorescence from the glass surface was collected through a dichroic beamsplitter and a bandpass filter optimized for coumarin (FF01-485/70-25 and Di01-R405, Semrock). The images were captured on a cooled EMCCD camera (1002 pixels × 1004 pixels; 8 μm × 8 μm per pixel; iXon+ 885, Andor Technology) using a 200 ms exposure and a 30 linear EM gain. To avoid photobleaching of the coumarin dye, each image was taken at a different field of view over the course of 2−250 s. Proper focusing was maintained using an autofocus system (Definite Focus, Carl Zeiss).

To extract the kinetic parameters, we used a Michaelis−Menten model for labeled nucleotide (represented by dNTP for the sake of simplicity) incorporation by a DNA polymerase (Pol):

$$\text{Pol}\cdot\text{DNA}_n + \text{dNTP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Pol}\cdot\text{DNA}_n\cdot\text{dNTP} \overset{k_2}{\rightarrow} \text{Pol}\cdot\text{DNA}_{n+1} + \text{PP}_i$$

where Pol·DNA$_n$ is the polymerase−primer/template complex, Pol·DNA·dNTP is the complex of Pol·DNA and dNTP, and Pol·DNA$_{n+1}$ is the one-base extension product. The binding and dissociation of nucleotides are known to be much faster than the chemical reaction step and are thought to reach rapid equilibrium,[3] with a dissociation constant (K_d) equal to ([Pol·DNA$_n$][dNTP])/[Pol·DNA$_n$·dNTP].

The rate of product formation is then given by

$$d[\text{Pol}\cdot\text{DNA}_{n+1}]/dt = (k_2/K_d)[\text{Pol}\cdot\text{DNA}_n][\text{dNTP}] \quad (1)$$

Because the total polymerase species is conserved, we have

$$[\text{Pol}\cdot\text{DNA}]_{\text{total}} = [\text{Pol}\cdot\text{DNA}_n] + [\text{Pol}\cdot\text{DNA}_n\cdot\text{dNTP}] + [\text{Pol}\cdot\text{DNA}_{n+1}] \quad (2)$$

Using eq 2 and assuming a large excess of nucleotide is used ([dNTP] unchanged), we can solve eq 1 to obtain the concentration of extension product as a function of time:

$$[\text{Pol}\cdot\text{DNA}_{n+1}] = [\text{Pol}\cdot\text{DNA}]_{\text{total}}\{1 - e^{-[k_2[\text{dNTP}]/(K_d+[\text{dNTP}])]t}\} \quad (3)$$

[Pol·DNA$_{n+1}$] is linearly proportional to the fluorescence intensity. Its values at the different time points were calculated by integrating the full field of view of the time-lapse images using MATLAB (Mathworks). The values were plotted as a function of time and fitted to eq 3 using a nonlinear least-squares regression method to extract the observed reaction rate constant (k_{obs}). The data and fits are shown in Figure 2. The curves are smooth, and the fits are excellent with adjusted R^2 values of >0.98.

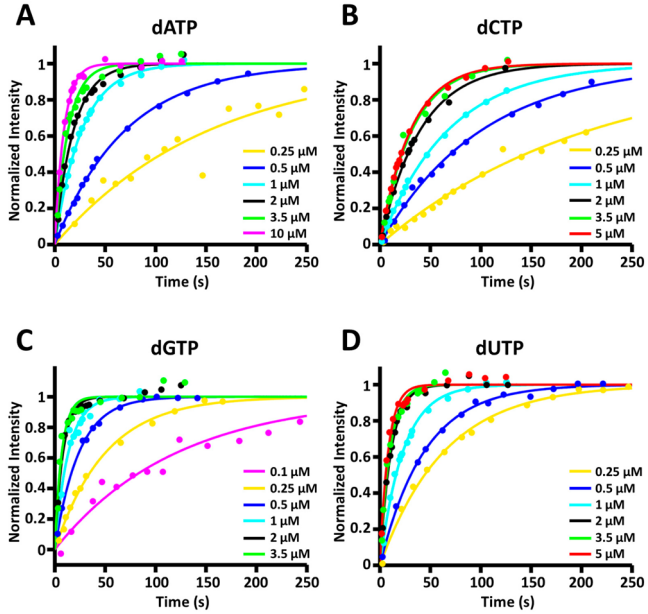

Figure 2. Bst DNA polymerase incorporation kinetics of coumarin-labeled dNTPs at a series of concentrations. Each data curve was fitted to an exponential in the form $a(1 - e^{-k_{obs}t}) + c$ and then offset and normalized.

In eq 3, the observed reaction rate constant is given by

$$k_{obs} = k_2[\text{dNTP}]/(K_d + [\text{dNTP}]) \quad (4)$$

So we plotted the k_{obs} values as a function of nucleotide concentration and fitted the curves using eq 4 to determine the dissociation constant (K_d) and turnover number (k_2). As shown in Figure 3, the data can be fitted very well with adjusted R^2 values of >0.99.

The K_d and k_2 values for the four coumarin-labeled nucleotides are listed in Table 1. Error values represent the 95% confidence bounds for the parameter fits. The turnover numbers measured at 25 °C vary from ∼0.05 to ∼0.24 s^{-1}, which are lower than the turnover rates for natural nucleotides with other polymerases, usually in the range of tens to hundreds of nucleotides per second. This is expected because the reactions were conducted below the optimal temperature of

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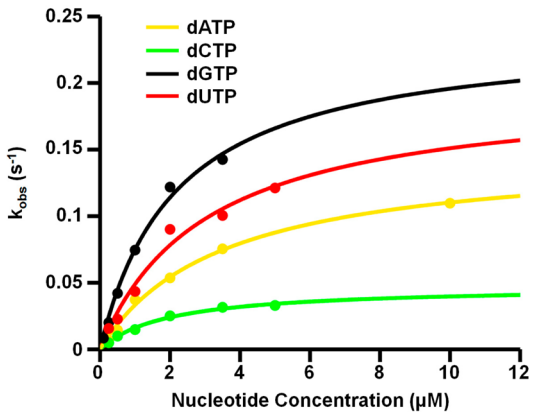

Figure 3. Observed reaction rate constants as a function of the concentrations of four coumarin-labeled nucleotides.

Table 1. Turnover Rates and Dissociation Constants

nucleotide	k_2 (s^{-1})	K_d (μM)
coumarin-dATP	0.15 ± 0.02	3.5 ± 1.3
coumarin-dGTP	0.24 ± 0.06	2.2 ± 1.1
coumarin-dUTP	0.20 ± 0.08	3.0 ± 2.5
coumarin-dCTP	0.047 ± 0.008	2.0 ± 0.8

Bst DNA polymerase (~65 °C) and the chemical modification could also affect the rates. Dissociation constants range from ~2 to 3.5 μM within K_M values reported for other polymerases and native nucleotides.[15] K_d equals K_M if rapid equilibrium of nucleotide binding and dissociation is assumed ($k_{-1} \gg k_2$).

Our method provides an alternative to the stopped-flow and quench-flow methods for measuring incorporation kinetics of labeled nucleotides by DNA polymerases. We use a microfluidic flow cell for the rapid initiation of the reactions and TIRF for direct real-time monitoring of the reactions on the surfaces, eliminating the needs for stopped-flow or quench-flow apparatus and subsequent analysis by electrophoresis. Using our setup with an imaging window of 80 μm × 80 μm and a linear fluid-flow velocity of 100 mm/s, a reaction can be initiated in <1 ms, a performance on par or better than that of stopped-flow and quench-flow methods. TIRF allows good signal to background kinetics measurements with up to 10 μM fluorescently labeled nucleotides in the bulk solution. Optimization of the TIR illumination angle may allow for the use of higher concentrations of labeled nucleotides. To prevent photobleaching of the fluorescent dyes, we acquired the time-lapse images at a different field of view for each image. Because the imaged area is relatively small and very uniform, the field-to-field signal intensity variation was not considered.

An interesting variation of our technique is to use a mixture of a native unlabeled nucleotide and its corresponding labeled nucleotide and to perform the reactions with various ratios and concentrations of the labeled and native nucleotides. We have shown that it is feasible to extract the kinetic parameters for both natural and labeled nucleotides simultaneously (M. T. Walsh and X. Huang, manuscript to be published). The information can then be used to predict the ratio of labeled to native nucleotides required to incorporate a desired percentage of the labeled nucleotide to implement a novel sequencing strategy called natural sequencing by synthesis (nSBS), in which a small fraction of a nonterminating fluorescently labeled nucleotide is incorporated along with the corresponding natural nucleotide in the cyclic DNA sequencing by a synthesis process.[16] In summary, our method allows the rapid and automated measurements of the nucleotide incorporation kinetics of DNA polymerases, which will be very useful for the mechanistic studies of DNA polymerases and for implementing and improving the current generation of DNA sequencing technologies that rely on the incorporation of labeled nucleotides by DNA polymerases.[17−20]

■ ASSOCIATED CONTENT

Supporting Information

Materials, methods, tables, and figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00269.

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Notes

The authors declare no competing financial interest.

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