Label-free optical detection of single enzyme-reactant reactions and associated conformational changes

Eugene Kim,*† Martin D. Baaske,*† Isabel Schuldes,†‡ Peter S. Wilsch,† Frank Vollmer*§

Monitoring the kinetics and conformational dynamics of single enzymes is crucial to better understand their biological functions because these motions and structural dynamics are usually unsynchronized among the molecules. However, detecting the enzyme-reactant interactions and associated conformational changes of the enzyme on a single-molecule basis remains as a challenge to established optical techniques because of the commonly required labeling of the reactants or the enzyme itself. The labeling process is usually nontrivial, and the labels themselves might skew the physical properties of the enzyme. We demonstrate an optical, label-free method capable of observing enzymatic interactions and associated conformational changes on a single-molecule level. We monitor polymerase/DNA interactions via the strong near-field enhancement provided by plasmonic nanorods resonantly coupled to whispering gallery modes in microcavities. Specifically, we use two different recognition schemes: one in which the kinetics of polymerase/DNA interactions are probed in the vicinity of DNA-functionalized nanorods, and the other in which these interactions are probed via the magnitude of conformational changes in the polymerase molecules immobilized on nanorods. In both approaches, we find that low and high polymerase activities can be clearly discerned through their characteristic signal amplitude and signal length distributions. Furthermore, the thermodynamic study of the monitored interactions suggests the occurrence of DNA polymerization. This work constitutes a proof-of-concept study of enzymatic activities using plasmonically enhanced microcavities and establishes an alternative and label-free method capable of investigating structural changes in single molecules.

INTRODUCTION

Enzymes fulfill a plethora of metabolic functions in all living organisms. In many cases, enzymatic activity is closely connected to changes in the enzymes’ conformation often involving the transition through multiple substrates. One of the most important and, perhaps, most studied enzymes is DNA polymerase, which is present in all cells and responsible for replicating genetic information. Outside of the actual metabolisms, it is used for important biological applications, such as polymerase chain reaction (PCR) and DNA sequencing. The enzymatic activity of DNA polymerase involves multiple steps, such as the binding of primer-hybridized template DNA, insertion of a deoxynucleoside triphosphate (dNTP), and incorporation of dNTP, thereby extending the strand by 1 nucleotide (nt). Each step of such a catalytic process is accompanied by conformational changes of the DNA polymerase. These transitions, together with the corresponding reaction pathways, have an intrinsically transient nature and have been vastly studied via single molecule–based techniques. The most widely used method is perhaps single-molecule Förster resonance energy transfer (FRET), which resolves the dynamics of DNA/polymerase interactions and the associated structural changes by measuring distances between labels attached to specific polymerase domains or DNA strands (1–5). Despite its great contribution to the extension of knowledge on the reaction mechanisms of DNA polymerase, this method intrinsically requires chemical modification of the enzyme to attach labels; hence, it can cause the studied enzyme to deviate from its natural kinetics. Inherent physical processes, such as photobleaching and large background signals, also limit the applicability of FRET (6). As a result, there is a great demand for label-free methodologies that can potentially broaden the scope of single-enzyme studies and complement the information obtained using label-based methods.

Here, we establish a label-free optical sensor platform capable of monitoring the kinetics and conformational dynamics of single enzymes, thus extending our previous bulk-sensing approach (7). We show that the kinetics of single-molecule DNA/polymerase (sm-DNA/Pol) interactions and the related conformational transitions of the polymerase can be studied using plasmonically enhanced whispering gallery mode (WGM) microcavity sensors (8–15). Our sensor recognizes conformational changes and the motion of single enzymes as shifts of the cavity’s optical resonance wavelength induced by the perturbation of the highly localized electric field at the tips of nanorods (NRs). The magnitude and sign of these shifts are proportional to the change in the electric field intensity integrated over the volume of the molecule. For an increasing (decreasing) integrated intensity, the induced resonance shift is toward longer (shorter) wavelengths. This can be applied for the study of molecular kinetics as follows. When a molecule enters the enhanced electric near field in the vicinity of the NRs, it causes a spectral red shift with an increasing magnitude as it moves toward the field’s intensity maximum. This is followed by a blue shift with the same magnitude when the molecule moves away from the NRs and leaves the near field completely. This concept also holds true when a molecule that is immobilized on the NRs changes its shape (that is, conformational state) in such a manner that the change in the volume-integrated field intensity is sufficient to cause recognizable spectral shifts of the WGM’s position in either direction. Here, we use these mechanisms to probe sm-DNA/Pol interactions with two different approaches: immobilization of DNA on the NRs for the study of sm-DNA/Pol interaction kinetics and immobilization of polymerase on the NRs for the observation of specific conformational transitions accompanied by its interaction with DNA molecules. In both cases, the statistical analysis of our sensor’s signals allows us to discern the different activity levels of three polymerase species: the Klenow fragment

Kim et al., Sci. Adv. 2017; 3: e1603044 29 March 2017

1 of 8
of Escherichia coli DNA polymerase I (KF) and DNA polymerases from Thermus aquaticus (Taq) and Pyrococcus furiosus (Pfu). We also study the sm-DNA/Pol interaction kinetics and associated conformational changes with respect to the type of DNA [primer/template DNA (ptDNA) and single-stranded DNA (ssDNA)], temperature, and the presence of dNTPs.

RESULTS AND DISCUSSION
Method for monitoring single-molecule DNA/polymerase interactions
The experimental setup used for monitoring interaction kinetics between polymerase and DNA is depicted in Fig. 1A. A fused silica microsphere with a diameter of ~80 to 100 μm serves as a WGM resonator and is placed inside a liquid sample cell made of polydimethylsiloxane. A Peltier element, which is attached to the wall of the sample cell, allows for temperature regulation of the liquid. WGMs are excited via frustrated total internal reflection of a wavelength-tunable laser beam (λc ≈ 642 and 780 nm) focused on the surface of a prism. The resonance wavelengths of WGMs are then determined from the transmission spectra obtained by sweeping the laser wavelength with a frequency of 50 Hz using a modified centroid method (14).

Single-molecule sensitivity can then be achieved by using the plasmonic near-field enhancement provided by gold NRs, whose longitudinal surface plasmon resonances match the laser’s wavelength (12–17). For this, the NRs are immobilized on the resonator (see Methods for the chemical protocols) in a directly monitored process, allowing one not only to count the number of deposited NRs but also to determine if their long axes are aligned reasonably parallel to the polarization of the electric field via the binding-induced linewidth broadening and wavelength shifts of the monitored WGM (section S1). The local perturbations of the electric field near the NRs, as induced by sm-DNA/Pol interactions, can then be recognized as shifts Δλ in the spectral position of WGMs (Fig. 1B). As aforementioned, the magnitude and sign of these shifts are proportional to the changes in the electric field intensity integrated over the volume occupied by the molecule vmo(t) at the times t1 and t2 = t1 + Δτ (where Δτ = 20 ms is the time between two laser sweeps) and to the molecule’s polarizability in excess to the medium αe (assuming a constant and isotropic molecular polarizability) (9, 10, 18)

\[
\Delta \lambda \propto \alpha_e \left( \int_{v_m(t_2)} |E(r)|^2 dV - \int_{v_m(t_1)} |E(r)|^2 dV \right) = \alpha_e (I(t_2) - I(t_1))
\]

where \(E(r)\) denotes the unperturbed electric field in the absence of the polymerase. However, this does not consider that the process of sweeping the laser over the spectral range occupied by the resonant mode (indicated as \(\lambda_m\) in Fig. 1B) itself requires a certain time \(\tau_m\). Consequently, each experimentally measured volume-integrated intensity \(I_{exp,k}\) for the kth sweep of the laser originates from an averaging process

\[
I_{exp,k} = \bar{I}_k = \left( \tau_m \right)^{-1} \int_{t_0}^{t_0+\tau_m} I(t) dt
\]

![Fig. 1. Methods for the detection of sm-DNA/Pol interactions. (A) Schematic of the prism-based microcavity sensor setup. The inset shows an image of NR scatterers bound to the equatorial plane of a microsphere. (B) Typical transmission spectra showing a WGM (Lorentzian dip) before (blue) and during (red) a DNA/polymerase interaction. (C) Conceptual representation of the two different approaches used for monitoring DNA/polymerase interactions (immo-DNA and immo-Pol scheme) and (D) the corresponding resonance traces, exhibiting spike signals caused by the respective DNA/polymerase interactions.](image-url)
where \( t_0 \) is the time in which the excitation of the mode begins. As a result, the experimentally obtained shifts are

\[
\Delta \lambda_k \propto \alpha_e (I_k - I_{k-1}) = \alpha_e \Delta I_k
\]

(3)

Therefore, our sensor can only recognize molecular interactions that keep the analyte molecules confined temporally on the order of \( \tau_m \approx 200 \mu s \) and spatially within the plasmonic hotspots (that is, near the NR tips). Molecular processes shorter than \( \tau_m \) but occurring repeatedly during \( \tau_m \) can also be recognized but with reduced magnitudes, whereas one-time events shorter than \( \tau_m \) (for example, freely diffusing analyte molecules near the hotspots) are unlikely to be recognized as their \( \Delta I_k \) is significantly lower (section S9) (14). To study the sm-DNA/Pol interactions, we take two approaches: The first approach (Fig. 1C, top) is based on the polymerase interacting with DNA strands immobilized on the NRs (henceforth referred to as the immo-DNA scheme). In this case, the shifts occur because of the changes in \( \Delta \lambda \) as polymerase molecules are attached and detached from the DNA strands, consequently moving in and out of the areas with high field intensities (Fig. 2, A and D). For the second approach (Fig. 1C, bottom), the polymerase molecules are immobilized on the NRs (henceforth referred to as the immo-Pol scheme), and the observed shifts are caused by changes in \( I \) due to the polymerase changing its conformational states accompanied by its interaction with DNA strands (Fig. 2, B, C, and E). Using both approaches, we obtain similar transient signal patterns (so-called “spikes”) arising from the sm-DNA/Pol interactions (Fig. 1D). These are composed of an initial red shift of the resonance position as a molecular interaction starts and a consequent return to the unperturbed mode position (blue shift) as the interaction ceases. Each individual spike exceeding the wavelength noise \( \sigma \) by at least three times is found and extracted using a spike detection algorithm (14). This algorithm also removes background drifts and returns the average and maximum shifts (\( \Delta \lambda \) and \( \Delta \lambda_{\text{max}} \), respectively) and the spike duration (\( \Delta t \)). In line with the proofs of the single analyte nature demonstrated in our previous studies (13–15), we have found that the detected spikes from the sm-DNA/Pol interactions originate from a Poisson process, and their detection rates scale linearly with the analyte concentrations (section S2). However, we would like to note that the presented single-molecule proofs do not indicate that only one receptor molecule (the immobilized reactant) was monitored overall because the spikes can originate from an ensemble of receptor molecules immobilized on the NRs. Nonetheless, the statistical proof confirms that each individual spike originates from a single receptor interacting with a single analyte molecule, whereas previous or later spikes may originate from a different receptor.

To further elaborate on the sensor’s response, we have performed finite element simulations to obtain the near-field intensity \( I \) of the electric field in the proximity of NRs. For this, we used simplified geometry for the polymerase consisting of two moving arms and a stationary bottom with the size parameters obtained via x-ray crystallography (19). Specifically, we compare the changes in \( I \) that are associated with the movement of the polymerase (Fig. 2, A and D) as a correspondence to the immo-DNA scheme. For the immo-Pol scheme, the changes in \( I \) depending on the variations of angular spread between the thumb and the finger domain of the polymerase were compared at two different immobilization positions (Fig. 2, B, C, and E). The near-field intensity exhibits a highly inhomogeneous distribution within the volume of the polymerase and rapidly decays with increasing distance from the NR’s tip. Consequently, \( I \) decreases significantly as the gap \( \Delta d \) between the NR and the polymerase increases on the scale of a few nanometers (Fig. 2D). Furthermore, \( I \) generally increases as the angle \( \theta \) between the two arms of the polymerase increases, whereas its absolute value and \( \theta \) dependency largely vary for different immobilization locations (Fig. 2E). In addition, we observed that for a different bound position of the polymerase, an increment of the angle \( \theta \) could also lead to a decrease in \( I \). This indicates that the WGM shift induced by the same conformational change of the polymerase (that is, the same \( \theta \)) can exhibit a different magnitude and a different sign of the shift. However, in the experiments, only the spikes toward longer wavelengths were observed (Fig. 1D).

**Interaction kinetics of different DNA polymerase species**

By using the immo-DNA approach (Fig. 1C, top), we experimentally inspect the interaction kinetics of two different polymerase species, Taq and KF, at a temperature (\( T \)) of \( \approx 293 \) K. Both Taq and KF are

---

**Fig. 2. Near field–based transduction mechanism.** (A to C) Spatial distributions of the near field’s intensity and the parameters associated with the respective movement of the polymerase volume. (A) Distance between the NR and the polymerase. (B) Changing angle between both arms with fixed base. (C) Changing angle between both arms with fixed left arm. a.u., arbitrary units. (D and E) Dependence of the volume-integrated intensity \( I \), normalized to \( V_{\text{nr}} \), on the parameters associated with (A) to (C).
expected to show apparent differences in their kinetic behavior because their enzymatic activity is optimal at distinctly different temperatures \((T_{opt})\) of 348 to 353 K and 310 K, respectively. Representative resonance wavelength traces for both species are shown in Fig. 3A.

In the case of Taq, we do not observe any change in noise level associated with either the presence or the absence of Taq and ptDNA. However, as for KF, the noise level rises once KF is added and increases even further after DNA is immobilized on the NRs. The former noise increase may be attributed to the unspecific short and reversible attachment of KF to the NRs, whereas the latter might originate from KF interacting with ptDNA molecules bound to locations on the NRs with low field intensities. Distinct spikes are observed only if ptDNA is immobilized on the NRs and if Taq or KF is present in solution, thus confirming the specificity of the monitored sm-DNA/Pol interactions (section S3). Furthermore, the expected difference in the kinetic behavior of Taq and KF is directly evident by comparing the spike magnitude and duration distributions obtained for both species (Fig. 3, B and C). The spike amplitudes found for Taq/ptDNA/dNTP interactions exhibit an exponentially decaying distribution (Fig. 3B, top), with 53% of the events populating the first bin above the 3σ limit. In contrast, the spike amplitudes obtained for KF/ptDNA/dNTP interactions have a significantly broader distribution exhibiting a clear peak at Δλc, well in excess of 3σ (Fig. 3B, bottom). This stark difference in the distributions is, at first, surprising because one would expect higher spike amplitudes for Taq polymerase because of its larger molecular mass (98 kDa) compared to KF (68 kDa). However, the spike magnitude is the result of a temporal averaging process (Eqs. 2 and 3) and, therefore, should be seen in conjunction with the spike duration because events shorter than \(\tau_m\) are recognized with a reduced shift magnitude. We find the spike durations \(\Delta t\) associated with Taq/ptDNA interactions (Fig. 3C, left) to be significantly shorter than those originating from KF/ptDNA interactions (Fig. 3C, right). Correspondingly, both species also yield distinctly different off-rates \((k_{off})\) of 47 and 23 s\(^{-1}\) [extracted via fitting of \(N(\Delta t) \sim e^{-k_{off}\Delta t}\) to the respective distributions]. The fact that the experiments were performed at 293 K, a temperature rather close to the optimal temperature for KF but well below that for Taq, indicates that there is a correlation between our sensor’s signal and the activity of the monitored enzyme. However, it is worthwhile to note that the off-rates, which are extracted from the Δt distributions, require careful interpretation because they are not necessarily equivalent to the dissociation rates between ptDNA and the polymerase. They rather reflect how long the polymerase resides within the plasmonic hotspots (that is, a period for which the value of \(I\) is high enough to be recognized) while it interacts with a DNA strand. This means that \(I\) can drop below the recognition threshold before the actual DNA/Polymerase interaction has ended because the polymerase moves along the overhanging template strand and away from the NR’s surface. In addition, the polymerase will start its activity at the end of the primer strand, which is \(\approx 8\) nm away from the NR’s surface, thus not reaching the maximum possible field overlap (compare Fig. 2D). Consequently, our values for \(k_{off}\) exceed the dissociation rates reported in other studies \([2, 20, 21]\) by at least one order of magnitude because we recognize only a small fraction of the actual reaction process along the entire template strand. Thus, it is evident that by using the immo-DNA approach, the amount of information that is directly obtainable is limited, although it still allowed us to recognize differences in the interaction kinetics of KF associated with ssDNA and ptDNA/dNTP interactions (section S5). Hence, we use the approach of immobilizing the polymerase on the NRs (the immo-Pol scheme) in the following studies of sm-DNA/Polymerase interactions.

---

**Fig. 3.** sm-DNA/Polymerase signals using immo-DNA scheme. (A) Example resonance traces exhibiting spike patterns caused by Taq (top; blue) and KF (bottom; maroon) polymerase/DNA interactions and the different noise levels found for ptDNA-functionalized NRs (maroon), unfunctionalized NRs (green), and in the absence of KF (light blue). (B) Distributions of the average spike amplitudes Δλc and (C) durations Δt obtained for Taq (blue) and KF (red) interacting with ptDNA in the presence of dNTP. The concentrations of Taq, KF, and dNTP were kept to \(=200\) nM, 200 nM, and 50 μM, respectively.
**Conformational transitions of Pfu polymerase at various temperatures**

The above results suggest a possible correlation between our sensor signal (namely, the spike amplitudes and durations) and the activity of the monitored enzymes. Nonetheless, it is challenging to precisely determine the physical process associated with the signals because conformational changes and the motion of the whole enzyme cannot be directly separated from the immo-DNA scheme. To exclude the polymerase motion’s contribution to the signals, we have performed experiments with polymerase immobilized on the NRs (immol-DNA scheme as shown in Fig. 2B). For this, we selectively use Pfu polymerase because it maintains its enzymatic activity even when immobilized on the NR surface. Experimental data confirming its activity and successful immobilization on the NRs are provided in sections S4 and S6.

While monitoring the sm-DNA/Pol interactions in this scheme, we recognize spike events similar to the ones observed using the previously discussed immo-DNA scheme. Furthermore, the rates at which these spikes occur exhibit a linear dependence on the ptDNA’s concentration (fig. S2A), whereas no spikes were recognized in the absence of ptDNA (fig. S3A, bottom) and when the NRs were not modified with Pfu polymerase, thus confirming that the spikes originate from individual ptDNA/Pol interactions.

To further elaborate on the mechanism behind these spikes, we also monitored DNA/Pol interactions for ptDNA strands with different lengths (section S7), which yielded no significant difference in the observed spike amplitudes despite a 3.3-fold increase in the ptDNA’s molecular mass. On the basis of this finding and together with the fact that the immobilized polymerase has a significantly larger volume (as compared to the ptDNA) and, in turn, occupies a larger fraction of the sensing volume (Fig. 2), we believe that the spike signals cannot solely originate from ptDNA molecules directly perturbing the field close to the NRs but may rather be induced by conformational changes of the ptDNA/Pfu complex accompanied by the initial incorporation of ptDNA. However, we would like to note that relating the patterns of individual spikes with transitions between specific conformational states is difficult because, in the current sensing scheme, even a single point inside one spike is the result of an averaging process over a period \(\tau_{\text{win}}\) (Eq. 2), which may include multiple transitions between conformational substates.

The relation between the recognized spikes and the conformational changes of the Pfu polymerase is further supported by their temperature dependence. The conformational changes of the thermophilic Pfu polymerase are closely linked to its enzymatic activity, which, in turn, is dependent on the ambient temperature and reaches its maximum in the 345 to 348 K range. Thus, we study how the durations and magnitudes of the ptDNA triggered spike signals change as we stepwise increase the ambient temperature toward this range while dNTPs are present in solution (Fig. 4). With respect to the spike amplitude distributions, we find that, with increasing temperature, the center of the peak position \(\Delta \lambda_c\) shifts toward higher amplitudes while their broadness also increases (Fig. 4A). On the one hand, this result is in line with our previous finding from the comparison of KF and Taq polymerase (Fig. 3) that the more active polymerase induces higher spike amplitudes (Fig. 4B). On the other hand, it further supports the premise that the recognized signals are not influenced by the ptDNA strands in terms of the direct addition of molecular mass to the Pfu molecules through binding. Furthermore, the fact that the peak center consistently shifts toward higher amplitudes as the temperature is adjusted toward values that favor the polymerase’s activity strongly suggests a relation between the spike amplitudes and conformational changes associated with the ptDNA/Pol interactions. These results specifically indicate that upon the incorporation of ptDNA,
the ptDNA/Pfu complex additionally undergoes a conformational change in such a manner that its time-averaged field overlap integral $I$ increases as compared to the moment of the initial ptDNA/Pfu interaction. Toward higher temperatures (where the enzyme has higher activity), this conformational transition that the ptDNA/Pfu complex undergoes evolves in such a way that the $\Delta I$ increases, which can be interpreted as an increase in the (time-averaged) magnitude of the conformational change.

Furthermore, the spike durations observed for the Pfu/ptDNA/dNTP interactions also become shorter as the temperature and, thus, the expected activity of the polymerase increase (Fig. 4C). This is in line with the expectation that the polymerase processes the ptDNA faster and consequently releases it earlier under more favorable ambient conditions.

We next compare the temperature-dependent Pfu/DNA interactions in the absence and presence of dNTPs (see Fig. 5). Independent of the presence of dNTPs, the peak centers of the spike amplitude distributions shift again toward higher amplitudes with increasing temperature, whereas the shifts are larger if dNTPs are present (Fig. 5A). Furthermore, we find that $\Delta \lambda_c \propto \epsilon^3$ (Fig. 4B), where the values for $A$ as determined from the corresponding Arrhenius plots (Fig. 5B) yield $-6.5 \times 10^3 \pm 0.9 \times 10^3$ K ($-1.7 \times 10^3 \pm 0.7 \times 10^3$ K) in the presence (absence) of dNTPs. The precise numerical values for $\Delta \lambda_c$ are listed in table S1. Here, the overall higher spike amplitudes that were found in the presence of dNTPs might be associated with the polymerase undergoing changes between additional conformational substrates accompanying the extension of the primer strand by possibly multiple dNTPs (the sensor’s time resolution is too low to resolve the incorporation of single dNTPs), yielding an increased $\Delta I$. The temperature dependence of the $k_{\text{off}}$ values as extracted from spike duration ($\Delta t$) distributions, however, displays a significant difference with respect to the presence of dNTPs. Although we find that $k_{\text{off}} \propto \epsilon^3$ for both cases, the sign of the values for $B$ as extracted from the corresponding Arrhenius plots (Fig. 5D) is different and $B$ values yield $-3.8 \times 10^3 \pm 0.6 \times 10^3$ K ($2 \times 10^3 \pm 1 \times 10^3$ K) in the presence (absence) of dNTPs. The result obtained in the presence of dNTPs is in line with what we had found before, namely, the increasing speed of the enzymatic process. However, in the absence of dNTPs, the spike durations increase with increasing temperature (Fig. 5C). This indicates that after the ptDNA/Pfu complex is formed, it remains in a certain conformation for a duration that increases as the temperature increases, until the ptDNA is eventually released again. This, in turn, implies that the ptDNA/Pfu complex is more stable at higher temperatures. From an enzymatic point of view, such a property is certainly desirable because, in this state, the polymerase is waiting for the arrival of dNTPs and a premature release of the ptDNA would be a waste of energy. These results, especially the distinctively different temporal behavior in the presence and absence of dNTPs, provide strong evidence that the

Fig. 5. Different conformational transitions in the presence and absence of dNTPs. (A) Comparison between average spike amplitude $\Delta \lambda_c$ distributions obtained for Pfu/DNA interactions in the absence (top) and presence (bottom) of dNTPs (50 μM) at 296 and 315 K. Arrhenius plots displaying the temperature dependence of (B) $\Delta \lambda_c$ and off-rates $k_{\text{off}}$ (D) found for Pfu in the presence and absence of dNTPs. (C) Change of the spike duration distributions at two different temperatures in the absence of dNTPs. (A) and (C) were obtained with the same Pfu/NR-modified microsphere, whereas (B) and (D) show data obtained with six different sensors.
monitored conformational changes accompanied by the Pfu/DNA complex formation involved the incorporation of nucleotides.

CONCLUSIONS
We have demonstrated our sensor’s capability to detect polymerase/DNA interactions linked to enzymatic activity on a single-molecule level by monitoring the kinetics and conformational transitions of DNA polymerases. Our results exhibit a clear correlation between our sensor’s signal and the enzymatic activity of three different types of polymerases at various ambient temperatures. Moreover, we have shown that the magnitude and duration of the conformational changes of the polymerase associated with DNA interactions vary with respect to the presence and absence of dNTPs. In this context, we have found a distinct difference in the temperature dependence of the enzymes’ kinetic behavior, providing strong evidence for the correlation of our sensor’s signal with enzymatic activity in the form of nucleotide incorporation.

Our results are potentially significant because monitoring enzymatic activity in a multiplexed and high-throughput fashion is a crucial requirement for next-generation sequencing. The fact that our approach is label-free and the sensor’s signals can be monitored in real time opens a new and direct way to determine conformational substates of various proteins without the necessity to mitigate label-associated background fluctuations. However, our sensor is intrinsically limited by our signal amplification method because the plasmonically enhanced near field may only probe a fraction of the enzyme’s volume. This limitation might become an advantage because it may, in turn, allow for the selective probing of certain protein subdomains. Furthermore, enzymatic kinetics can be easily tested with respect to diverse medium conditions, such as molecule concentrations, ionic strength, pH, and temperature. In this context, combining our method with label-based techniques would be promising when linked to the extensive knowledge already established via label-based methods, thus diversifying quantitative analysis. For example, in combination with FRET, our sensor would allow for probing of conformational changes beyond FRET’s spatial limitations yet take advantage of its selectivity. Moreover, the temperature dependence of polymerase/DNA interactions can be further investigated by optically driven local heating of the NRs, which may allow for fast switching of the enzymes’ activity and the corresponding interaction kinetics.

METHODS
All solutions without NRs were filtered with 0.1-μm syringe filters (Merck Millipore) before usage. The power that was coupled to microwaves was <0.1 mW overall, accounting for an average total energy of <20 nJ being coupled to the resonators per wavelength sweep.

The immo-DNA recognition scheme
Sensor assembly was conducted by adopting a modified version of the three-step wet-chemical procedure used in the study by Baaske et al. (13). First, cetyltrimethylammonium bromide–stabilized gold NRs with diameters of 10 nm and lengths of 35 nm (Nanopartz) were immobilized on the microresonator surface. For this, the NRs were injected into a sample cell holding about 0.5 ml of 100 mM NaCl solution at pH ≈ 1.6. This process was directly monitored, and individual NR binding events were classified as discrete steps in the resonance position and linewidth traces. Second, thiol-modified ssDNA ([5′-TTT-TCTCGTTGGGGTCCTTC-3′; Eurofins]) were conjugated to the adsorbed NRs. To cleave any disulfide bonds, we treated the thiolated DNA with 100 mM dithiothreitol and 100 mM NaCl for 30 min at room temperature before measurement (22). The NR-modified sphere was then immersed in a solution with 500 mM NaCl, 0.02% (w/w) SDS at pH ≈ 3, and 1 μM DNA (13, 23). The time spans required to produce a surface coverage that was sufficient to monitor sm-DNA/Pol interactions were in the range of 10 to 30 min, although longer reaction times may result in undesirably high DNA surface densities and hinder protein-DNA interactions. Last, in NEBuffer 2 (New England Biolabs Inc.), the initial pH of the solution was reduced to 6.7 by adding 2 mM HCl to maintain stable NR adsorption. If required, ssDNA was hybridized to ptDNA by injection of the template strand DNA (60 nt, CCGACAACCATCAACCCGGTCTGAGCCAGCCGGCTGTGAGCAGGTCTGACCCCAACGAGA), followed by the introduction of the desired amount of polymerase (that is, Taq and KF; New England Biolabs Inc.).

The immo-Pol recognition scheme
Sensor assembly was performed according to the following steps. First, polymerase-gold NR conjugates were prepared by mixing 2 μl of Pfu DNA polymerase (BioVision) from a stock solution (2.5 U/μl) with 5 μl of a solution containing citrate gold NRs (diameter, 25 nm; length, 49 nm; Nanopartz) at a concentration of 5.7 × 1011 nanoparticles/μl. Second, the surface of the freshly fabricated microsphere was functionalized with amidopropyltriethoxysilane (C3H7NO2Si; APTES) by immersing the microsphere in a 100-μl droplet of 2.5% (v/v) APTES for about 1 to 2 min. The binding of Pfu-NR conjugates to the microsphere was then performed in a sample chamber filled with PCR buffer. The PCR buffer contained 10 mM tris-HCl, 50 mM KCl, 1.5 mM MgCl2, and 0.001% (w/v) gelatin (Sigma-Aldrich). Last, the ptDNA used for the measurements was prepared by mixing the template and primer strands (1:1 molar ratio) in a solution containing 50 mM NaCl. This mixture was heated to 94°C in an Eppendorf incubator and cooled down to room temperature before use. The observation of ptDNA and immobilized Pfu polymerase was then undertaken in the PCR buffer.

Numerical analysis
Numerical simulations were performed using COMSOL Multiphysics (frequency-domain module). The polymerase was modeled as three rectangular parallelepipeds (6 nm × 4 nm × 5 nm for the moving arms and 2 nm × 5 nm × 5 nm for the stationary bottom) that are conjugated with two cylinders (2 nm × 5 nm) to maintain a constant volume while increasing the angle between the two arms. It was initially attached to the center of the gold NR’s tip, and this gold NR was modeled as a prolate circular cylinder with hemispherical end caps with dimensions of 25 nm × 49 nm. The refractive index of the surrounding medium (water) used for the simulation was 1.332, and the frequency-dependent refractive index values of gold were taken from the study by Johnson and Christy (24). The simulation domain was surrounded by a perfectly matched layer (PML) to absorb the outward-propagating radiation. To calculate the local field enhancement, we illuminated the entire domain with a plane wave at a pump wavelength of 642 nm. The polarization state of the incoming field was parallel to the long axis of the NR. With regard to meshing, a swept mesh was used to discretize the external and PML domain. Note that the NR and polymerase domains were meshed using free tetrahedral discretization, whereas the finer meshes were used in the NR and polymerase domains.
SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/3/e1603044/DC1

section S1. Real-time monitoring of the NR immobilization procedure
section S2. Single-molecule analysis
section S3. Control experiments
section S4. PCR experiments
section S5. KK interactions with ssDNA and ptDNA/dNTP
section S6. Signal comparisons between Pfu-immobilized NRS and bare NRS interacting with a WGM cavity
section S7. Pfu polymerase interactions with two different lengths of ptDNA
section S8. Additional information
section S9. Discussion on the detection of freely diffusing polymerase molecules
fig. S1. Time-traced WGM resonance positions (Δ) and linewidth broadening (|ΔT|) with step-like changes induced by binding of individual NRs (dimensions, 25 nm × 49 nm; λ_{det} = 642 nm).
fig. S2. Single-molecule statistics.
fig. S3. Control experiments.
fig. S4. Gel of the PCR with citrate NRs and Taq/Pfu polymerase.
fig. S5. Comparison of KK interactions associated with ssDNA and ptDNA/dNTP.
fig. S6. Comparison of transient changes in WGM response position and WGM linewidth as induced by NRs (before and after Pfu-surface functionalization) entering the WGM’s evanescent field.
fig. S7. Pfu-polymerase interactions with two different lengths of ptDNA strands.
fig. S8. Sketch of the model used to perform Monte Carlo simulations.
fig. S9. Probabilities of a polymerase molecule to be still located inside the detection volume after a certain duration of time as obtained from Monte Carlo simulations using different initial molecule positions.
fig. S10. Probabilities of detecting resonance shifts with amplitudes of 15 (black) and 30 (red) nm of perturbations with a certain duration as shifts above 3σ (solid lines) or σ (dashed lines) during one wavelength sweep of the laser (20 ms).
fig. S11. Zoom in showing probabilities P_{det} and P_{meas} plotted on the interval and for the cases where they overlap with nonzero values.
fig. S12. Zoom in showing P_{meas}.
table S1. Numerical values used in the fitting process of P_{meas} to P_{det} in fig. S2, alongside the respective coefficients of determination R^2.
table S2. Numerical values of the results shown in the Arrhenius plots of Fig. 5.

REFERENCES AND NOTES

1. S. Farooq, C. Fijen, J. Hohlbein, Studying DNA-protein interactions with single-molecule Förster resonance energy transfer. Proteoplasma 251, 317–332 (2014).
2. T. D. Christian, L. J. Romano, D. Rueda, Single-molecule measurements of synthesis by a DNA polymerase with base-pair resolution. Proc. Natl. Acad. Sci. U.S.A. 106, 21109–21114 (2009).
3. Y. Santos, C. M. Joyce, O. Potapova, L. Le Reste, J. Hohlbein, J. P. Torella, N. D. F. Grindley, A. N. Kapanidis, Conformational transitions in DNA polymerase I revealed by single-molecule FRET. Proc. Natl. Acad. Sci. U.S.A. 107, 715–720 (2010).
4. J. Hohlbein, L. Aigrain, T. D. Craggs, O. Bernek, O. Potapova, P. Shoolizadeh, N. D. F. Grindley, C. M. Joyce, A. N. Kapanidis, Conformational landscapes of DNA polymerase I and mutator derivatives establish fidelity checkpoints for nucleotide insertion. Nat. Commun. 4, 2131 (2013).
5. S. Y. Berezhna, J. P. Gill, R. Lamichhane, D. P. Millar, Single-molecule Förster resonance energy transfer reveals an innate fidelity checkpoint in DNA polymerase I. J. Am. Chem. Soc. 134, 11261–11268 (2012).
6. N. Bevan, S. Rees, Pharmaceutical applications of GFP and RFP. Methods Biochem. Anal. 47, 361–389 (2006).
7. E. Kim, M. R. Foreman, M. D. Baaske, F. Vollmer, Thermal characterisation of (bio) polymers with a temperature-stabilised whispering gallery mode microsensor. Appl. Phys. Lett. 106, 161101 (2015).
8. M. A. Santiago-Cordoba, S. V. Boriskina, F. Vollmer, M. C. Demirel, Nanoparticle-based protein detection by optical shift of a resonant microcavity. Appl. Phys. Lett. 99, 073701 (2011).
9. S. I. Shopova, R. Rajmangal, S. Holler, S. Arnold, Plasmonic enhancement of a whispering-gallery mode biosensor for single nanoparticle detection. Appl. Phys. Lett. 98, 243104 (2011).
10. J. D. Swaim, J. Knittel, W. P. Bowen, Detection limits in whispering gallery biosensors with plasmonic enhancement. Appl. Phys. Lett. 99, 243109 (2011).
11. M. R. Foreman, F. Vollmer, Theory of resonance shifts of whispering gallery modes by arbitrary plasmonic nanoparticles. New J. Phys. 15, 083006 (2013).
12. V. R. Danthan, S. Holler, C. Barbre, D. Keng, V. Kolchenko, S. Arnold, Label-free detection of single protein using a nanoplasmonic-photonic hybrid microcavity. Nano Lett. 13, 3347–3351 (2013).
13. M. D. Baaske, M. R. Foreman, F. Vollmer, Single-molecule nucleic acid interactions monitored on a label-free microcavity biosensor platform. Nat. Nanotechnol. 9, 933–939 (2014).
14. M. D. Baaske, F. Vollmer, Optical detection of single atomic ions interacting with plasmonic nanorods in aqueous solution. Nat. Photonics 10, 733–739 (2016).
15. E. Kim, M. D. Baaske, F. Vollmer, In situ observation of single-molecule surface reactions from low to high affinities. Adv. Mater. 28, 9941–9948 (2016).
16. P. Zijlstra, P. M. Paulo, M. Orrit, Optical detection of single non-absorbing molecules using the surface plasmon resonance of a gold nanorod. Nat. Nanotechnol. 7, 379–382 (2012).
17. I. Amert, J. Prasad, A. Henkel, S. Schmachtel, C. Sönntsch, Single unlabeled protein detection on individual plasmonic nanoparticles. Nano Lett. 12, 1092–1095 (2012).
18. S. Biehs, M. Khoshsima, I. Teraoka, S. Holler, F. Vollmer, Shift of whispering-gallery modes in microspheres by protein adsorption. Opt. Lett. 28, 272–274 (2003).
19. S. W. Kim, D-U. Kim, J. K. Kim, L-W. Kang, H-S. Cho, Crystal structure of Pfu, the high fidelity DNA polymerase from Pyrococcus furiosus. Int. J. Biol. Macromol. 42, 356–361 (2008).
20. R. D. Kuchta, V. Mizrahi, P. A. Benkovic, K. A. Johnson, S. J. Benkovic, Kinetic mechanism of DNA polymerase I (Klenow). Biochemistry 26, 8410–8417 (1987).
21. A. Langer, M. Schürml, R. Stasser, H. Daub, T. Myers, D. Heindl, U. Rant, Polymerase/DNA interactions and enzymatic activity: Multi-parameter analysis with electro-switchable biosurfaces. Sci. Rep. 5, 12066 (2015).
22. S. R. Nicewanner Peña, S. Raina, G. P. Goodrich, N. V. Fedoroff, C. D. Keating, Hybridization and enzymatic extension of Au nanoparticle-bound oligonucleotides. J. Am. Chem. Soc. 124, 7314–7323 (2002).
23. D. Shi, C. Song, Q. Jiang, Z.-G. Wang, B. Din, A facile and efficient method to modify gold nanorods with thiocyanated DNA at a low pH value. Chem. Commun. 49, 2533–2535 (2013).
24. P. B. Johnson, R-W. Christy, Optical constants of the noble metals. Phys. Rev. B 6, 4370–4379 (1972).
25. L. P. Sun, S. Wang, Z. W. Zhang, Y. Y. Ma, Y. Q. Lai and J. Weng Interaction of gold nanoparticles with Pfu DNA polymerase and effect on polymerase chain reaction. IET Nanobiotechnol. 5, 20–24 (2011).
26. A. B. Johnson, K. A. Johnson, Exonuclease proofreading by human mitochondrial DNA polymerase. J. Biol. Chem. 276, 38097–38107 (2001).
27. A. J. Wowor, K. Datta, H. S. Brown, G. S. Thompson, S. Ray, A. Grove, V. J. LiCata, Thermodynamics of the DNA structural selectivity of the Pol I DNA polymerases from Escherichia coli and Thermus aquaticus. Biophys. J. 98, 3015–3024 (2010).

Acknowledgments: We thank C. Koch from the Biochemistry chair of Friedrich-Alexander University Erlangen-Nürnberg for providing access to the PCR instrument. E.K. and M.D.B. thank S. Vincent for his feedback on the manuscript. Funding: We acknowledge financial support from the Max Planck Society. Author contributions: E.K. and M.D.B. wrote the manuscript and simulated the development and performed the experimental setup. M.D.B. developed data analysis tools and supervised the experiment. P.S.W., I.S., and E.K. performed the experiment. F.V. supervised the entire project. All authors commented on the manuscript. Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in this paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 5 December 2016
Accepted 9 March 2017
Published 29 March 2017
10.1126/sciadv.1603044

Citation: E. Kim, M. D. Baaske, I. Schultes, P. S. Wilsch, F. Vollmer, Label-free optical detection of single enzyme-reactant reactions and associated conformational changes. Sci. Adv. 3, e1603044 (2017).