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GFP-like Fluorophores as DNA Labels for Studying DNA–Protein Interactions

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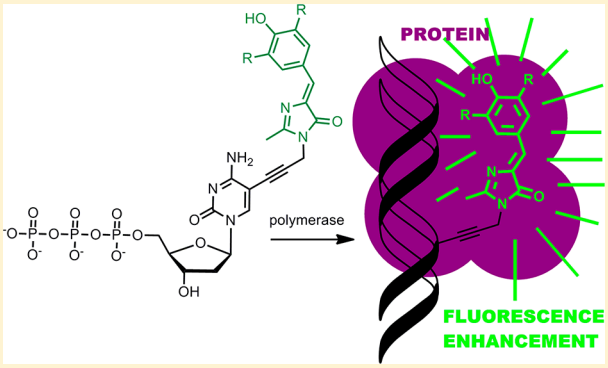
Supporting Information

ABSTRACT: GFP-like 3,5-difluoro-4-hydroxybenzylideneimidazolinone (**FBI**) and 3,5-bis(methoxy)-4-hydroxy-benzylideneimidazolinone (**MBI**) labels were attached to dCTP through a propargyl linker, and the resulting labeled nucleotides (**dC^{MBI}TP** and **dC^{FBI}TP**) were used for a facile enzymatic synthesis of oligonucleotide or DNA probes by polymerase-catalyzed primer extension. The **MBI**/**FBI**-labeled DNA probes exerted low fluorescence that was increased 2–3.2 times upon binding of a protein. The concept was demonstrated on sequence-specific binding of p53 to dsDNA and on nonspecific binding of single strand binding protein to an oligonucleotide. The **FBI** label was also used for a time-resolved experiment monitoring a single-nucleotide incorporation followed by primer extension by Vent(exo-) polymerase.



INTRODUCTION

Fluorescent labeling of biomolecules is an indispensable tool in chemical and molecular biology.[1] In nucleic acids, intrinsically fluorescent nucleobase analogues or nucleobase-linked fluorophores have been used and applied in hybridization assays,[2] single-nucleotide polymorphism typing,[3] monitoring of the polarity of the microenvironment,[4] and monitoring of interactions with a ligand.[5] However, very few examples of the use of fluorescent labeling of nucleic acids for direct detection of interactions with unlabeled proteins[6,7] or peptides[8] have been reported, although an analogous use in protein labeling for the detection of peptide–protein[9] or protein–DNA[10] interaction is well established. Very recently, we have developed[11] novel nucleotides bearing solvatochromic aminophthalimide fluorophores that were used as building blocks for the enzymatic synthesis of labeled DNA that enhanced fluorescence (up to 2-fold) upon binding of some proteins.

4-Hydroxybenzylideneimidazolinone (**HBI**) is a green-emitting fluorophore occurring in green fluorescent protein (GFP).[12] Unlike native GFP, **HBI** is very weakly fluorescent in denatured protein and as a free molecule prepared by chemical synthesis.[13] This phenomenon is caused by non-emissive energy dissipation caused by flexibility of bond rotation of the free **HBI** molecule. Relaxation to ground state by an emissive pathway requires planarity of a molecule accompanied by blocking of bond rotation. This condition is fulfilled in native GFP by specific interactions within the protein. The **HBI** fluorescence can also be induced by constrained blocking of bond rotation of free **HBI**, which was achieved by interactions of **HBI** derivatives with RNA.[14] Since the **HBI** requires suppression of subtle motions, 3,5-difluoro-4-hydroxybenzylideneimidazolinone (**FBI**) and 3,5-bis(methoxy)-4-hydroxybenzylideneimidazolinone (**MBI**) were found to be more useful than **HBI** for inducing fluorescence.[14] To the best of our knowledge, the only example[15] of a GFP-like fluorophore (2-hydroxybenzylidene-imidazolinone linked to 2′-OH of a ribose) was used for the labeling of oligonucleotides (ONs) showing large Stokes shifts.

Here we report on the synthesis of **MBI** and **FBI** conjugates with nucleoside triphosphates (dNTPs) linked via non-conjugate propargyl tether at C-5 of cytosine and their incorporation of DNA. We assumed that the **MBI** and **FBI** tags might be able to increase their fluorescence upon binding of the labeled DNA to a protein due to restricted rotation of the fluorophores. Such mechanism would be conceptually different from our previous solvatochromic labels.[11]

RESULTS AND DISCUSSION

Synthesis and Enzymatic Incorporation of MBI- and FBI-Labeled dNTPs. Our intended strategy for the synthesis of fluorescent ON or DNA probes was based on the polymerase incorporation of base-modified dNTPs[16] bearing the appropriate labels. The synthesis of the modified dNTPs

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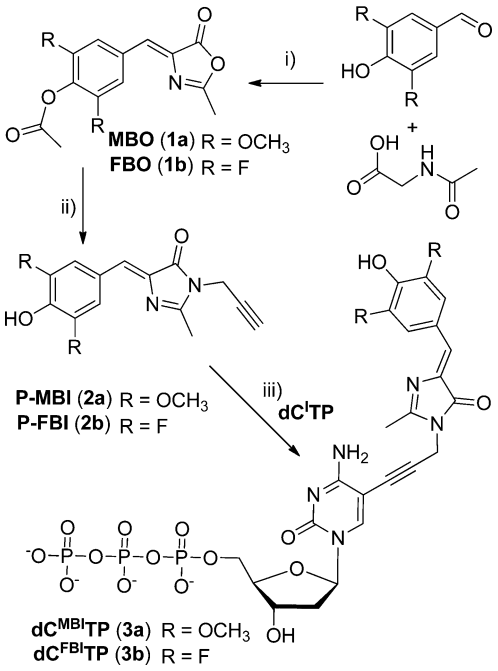
was envisaged through the direct Sonogashira cross-coupling[17] of halogenated dNTPs with a corresponding fluorophore-linked terminal acetylenes. These building blocks were prepared in analogy[14] to the synthesis of the fluorophores.

3,5-Dimethoxy- and 3,5-difluoro-4-hydroxybenzaldehydes were condensed with *N*-acetylglycine and cyclized by using acetic anhydride as a solvent to form oxazolinone derivatives **MBO** (**1a**) and **FBO** (**1b**) in good yields (Scheme 1). The

Scheme 1. Synthesis of MBI- and FBI-Labeled dNTPs[a]



[a]Reagents and conditions: (i) Ac_2O, (ii) propargylamine, K_2CO_3, EtOH (99%), (iii) $Pd(OAc)_2$, TPPTS, CuI, $EtN(i\text{-}Pr)_2$, water/acetonitrile (2:1).

oxazolinones were subsequently converted to propargylimidazolinones **P-MBI** (**2a**) and **P-FBI** (**2b**) by treatment with propargylamine in moderate yields (47% and 25%, respectively). The reaction also led to the formation of hydrolyzed byproduct, which were removed by extraction or by silica-gel chromatography (for **2b**). The desired **MBI**- and **FBI**-labeled dNTPs were prepared in one step by aqueous-phase Sonogashira coupling of **dC^{I}TP** with **P-MBI** (**2a**) or with **P-FBI** (**2b**) in the presence of palladium acetate, tris(3-sulfonatophenyl)phosphine (TPPTS), CuI, and the Hünnig base in water/acetonitrile (2:1) in moderate yields 21% and 28%, respectively (Scheme 1).

Both **dC^{MBI}TP** and **dC^{FBI}TP** were tested for the incorporation to DNA in a primer extension (PEX) experiment using KOD XL DNA polymerase and temp^{rnd16} as template (Figure 1, Table 1). The DNA polymerase was able to incorporate both **dC$^{M/FBI}$TP**s smoothly to form full length products, double-stranded 31-bp DNA bearing four modifications. In order to isolate single-stranded ONs (ssONs), the PEX was performed with a biotinylated template followed by magnetoseparation[22a] on streptavidin-coated magnetic beads and release under denaturating conditions.

Photophysical Properties of MBI- and FBI-Labeled DNA. Free **MBI** and **FBI** fluorophores emit very low fluorescence, as reported earlier.[14] Emission of both free

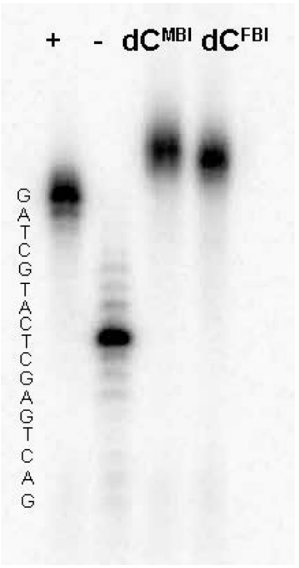


Figure 1. Incorporation of **dC^{MBI}TP** and **dC^{FBI}TP** using KOD XL DNA polymerase. PEX products contain four modifications using temp^{rnd16} template. Experiments are supplemented by positive control (+) (all natural dNTPs are present) and negative control (−) (absence of natural dCTP).

fluorophores is pH-dependent due to ionization of the phenol group. The pK_a value of **MBI** is 8, whereas the pK_a of **FBI** is 5.5 due to the −I effect of fluorine atoms.[14] **MBI** exerts a dual emission,[14] where the neutral form emits at 480 and the deprotonated form at 540 nm. On the other hand, a deprotonated form of **FBI** emits at 501 nm, while the neutral form is almost nonfluorescent, emitting at ca. 500 nm. To test the pH-dependence of the **MBI**/**FBI**-labeled DNA, we have prepared ssONs bearing either four **MBI** or four **FBI** modifications by primer extension and magnetoseparation (vide supra). Both the UV−vis and fluorescence spectroscopy of these modified ONs (Figures S1−S4 in Supporting Information) showed similar features as the free fluorophores, but the pK_a values of the fluorophores linked to ON probes were shifted to more basic due to interactions with the ON: **MBI** changed its pK_a from 8 to 9.3, while **FBI** changed the pK_a from 5.5 to 6.5 (Supporting Information). Both fluorophores still retain the desired low intensity of fluorescence in DNA.

Study of MBI- and FBI-Labeled DNA with DNA Binding Proteins. Similarly as in our previous study with solvatochromic labels,[11] the DNA−protein binding experiments were conducted on p53,[18] an important tumor suppressor[19] and cell cycle regulator, as an example of a sequence-specific protein binding to dsDNA and on single strand binding protein (SSB) as an example of a non-sequence-specific protein binding to ssONs. In addition, we tried to employ the labeled **dC^{FBI}TP** for a time-resolved monitoring of their incorporation by Vent(exo-) polymerase and of the further movement of the enzyme along the DNA.

Binding Study with p53 Protein. Interactions of DNA with p53 protein were studied using a 50-nt oligonucleotide (pex^{p53} prepared from temp^{p53} template using **dC^{MBI}TP** (**3a**) or **dC^{FBI}TP** (**3b**)) containing a 20-nt p53 recognition sequence and bearing 11 **dC^{MBI}** or **dC^{FBI}** fluorophores (6 within and 5 outside the recognition sequence). Bovine serum albumine (BSA), which does not bind DNA, was used as a control.

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Table 1. List of Oligo-2′-deoxyribonucleotides Used or Synthesized[a]

oligonucleotide	oligo-2′-deoxyribonucleotide sequence
prim^{rnd}[b]	5′-CATGGGCGGCATGGG-3′
temp^{rnd16}[b]	5′-**CTAGCATGAGCTCAGT**CCCATGCCGCCCATG-3′
ssDNA^{rnd16}[c]	5′-CATGGGCGGCATGGGA**C^{R}**TGAG**C^{R}**T**C^{R}**ATG**C^{R}**TAG-3′
prim^{p53}[b]	5′-GAATTCGATATCAAG-3′
temp^{p53}[b]	5′-**GACGGTATCGATAAGAGGCATGTCTAGGCATGTCT**CTTGATATCGAATTC-3′
prim^{vent}[b]	5′-CATGGGCGGCATGGGC-3′
temp^{vent}[b]	5′-**CTAGCATGAGCTCAG**GCCCATGCCGCCCATG-3′

[a]In the template (temp) ONs the segments forming a duplex with the primer are underlined, and the replicated segments are in bold. For magnetic separation of the extended primer strands, the templates were 5′-end biotinylated. Acronyms used in the text for primer extension products are analogous to those introduced for the templates (e.g., ssDNA PEX product ssDNA^{rnd16} was synthesized on temp^{rnd16} template, etc.). [b]Purchased oligonucleotide. [c]Model example using **dC^{R}** as modification.

The resulting **MBI**/**FBI**-labeled dsDNA (3 μM) was titrated by 0.5 or 1 equiv of p53 solution. The results revealed a perfectly ratiometric increase of intensity of **MBI** and **FBI** fluorescence upon binding of p53 protein (Figure 2 and Figure S7 in Supporting Information). The increase of fluorescence confirmed our assumption of a more hindered rotation of fluorophore due to the interaction with the protein. On the other hand, the addition of BSA did not increase the fluorescence. The 2.3-fold increase of **MBI** fluorescence intensity at 484 nm was also accompanied by an additional emission shoulder with maximum around 530 nm (even higher increase of ca. 2.6-fold at this wavelength), which originates from the ionized form of fluorophore formed either only in the excited state or stabilized by binding protein. The binding of p53 to **MBI**-labeled DNA therefore not only increases the emission but also causes a dual emission. On the other hand, binding of p53 to the **FBI**-labeled DNA exerted a slightly lower 2.1-fold increase of fluorescence at 496 nm.

Binding Study with SSB Protein. Interactions of SSB protein with **MBI**/**FBI**-labeled DNA were studied on a 31-nt ssON probe bearing four **MBI** or **FBI** modifications (prepared by PEX using pex^{rnd16} followed by magnetoseparation) in the sequence. The intensity of fluorescence was monitored upon the addition of SSB protein solution, and again BSA was used as a control. The experiments revealed a fully ratiometric 2.0-fold increase of fluorescence intensity of **MBI**/**FBI**-labeled ONs upon SSB binding at 489 and 501 nm, respectively (Figure 3). For MBI-modified ON, an additional emission band was observed at 535 nm, originating from the ionized form of the fluorophore. This additional emission band was even more significant than in the case of binding of p53 protein, showing a nearly 3.2-fold increase of fluorescence at this wavelength and even a 3.5-fold increase at 550 nm (Figure 3). This difference may indicate a different binding mode of the labeled ONs to SSB protein (according to the literature,[20] the ssON is coiled around the SSB tetramer). On the other hand, **MBI**- or **FBI**-labeled dsDNA (PEX product of pex^{rnd16} without magnetoseparation) exhibited very low increase of fluorescence upon SSB binding, indicating a priority of binding of SSB protein to ssDNA (Figures S5 and S6 in Supporting Information).

Monitoring of Incorporation of a dNTP and Primer Extension by Vent(exo-) DNA Polymerase. The last goal was to use the GFP-like labels for a time-resolved study of an enzymatic reaction, i.e., incorporation of a modified dNTP and movement of the polymerase along the DNA. For this purpose, only the **FBI** label was selected due to its simple emission behavior. The experiment was performed in two stages. At first, a single incorporation of the labeled **dC^{FBI}TP** into the primer was studied using an equimolar amount of Vent(exo-) DNA polymerase (Figure 4). The fluorescence spectra were measured immediately and then every minute after the addition of the **dC^{FBI}TP** until the change of fluorescence was completed. In the second stage, a mixture of natural dNTPs was added, which enabled the enzyme to continue the PEX and finish the DNA synthesis. Again, the fluorescence spectra were measured every minute until the change had finished. Figure 4d shows the expected increase of fluorescence upon the incorporation of the **dC^{FBI}TP** when the polymerase remains bound to the active site and thus interacts with the **FBI** label. The reaction and equilibrium of polymerase binding takes ca. 8 min to reach the maximum fluorescence enhancement (ca. 2.1-fold). Then, after the addition of the natural dNTPs (Figure 4e), the polymerase continued the synthesis of the DNA and moved along the duplex, releasing the **FBI** fluorophore. This caused a rapid decrease of fluorescence which reached the original low level within ca. 2 min. This experiment enables a direct monitoring of an enzymatic incorporation of a nucleotide and the following primer extension using an unmodified enzyme (this arrangement is much easier than the FRET techniques requiring labeling of both components[21]).

CONCLUSIONS

GFP-like hydroxybenzylideneimidazolinone labels **MBI** and **FBI** were studied as potential DNA labels for the studying of interactions with proteins. **MBI**- and **FBI**-labeled dCTPs (**dC^{MBI}TP**, **dC^{FBI}TP**) were prepared by aqueous Sonogashira coupling of propargyl-**MBI** or -**FBI** with halogenated **dC^{I}TP** in moderate yields. Both modified **dC$^{M/FBI}$TP**s were good substrates for Vent(exo-) and KOD XL polymerases and were incorporated to DNA by primer extension, enabling a simple synthesis of ssONs or dsDNA probes containing several site-specific **MBI** or **FBI** labels attached at the C-5 position of cytosine(s). Both fluorophores and the labeled DNA showed low fluorescence, which increased by 2.0−3.5 times upon binding of a protein to the ON or DNA probe due to hindered rotation of the fluorophores. The concept was proved both on a p53 to a dsDNA containing a specific recognition sequence and on a non-sequence-specific binding of SSB to ssON. The **FBI** label exerted simple increase of fluorescence at the same wavelength, whereas the **MBI** label showed a dual fluorescence with an additional emission band (at 530 or 535 nm) due to phenol deprotonation. Studying and monitoring of other DNA-binding proteins will continue in our laboratory.

FBI fluorophore was also successfully applied for a time-resolved detection of a single nucleotide incorporation by DNA polymerase (increase of fluorescence) and for the observation

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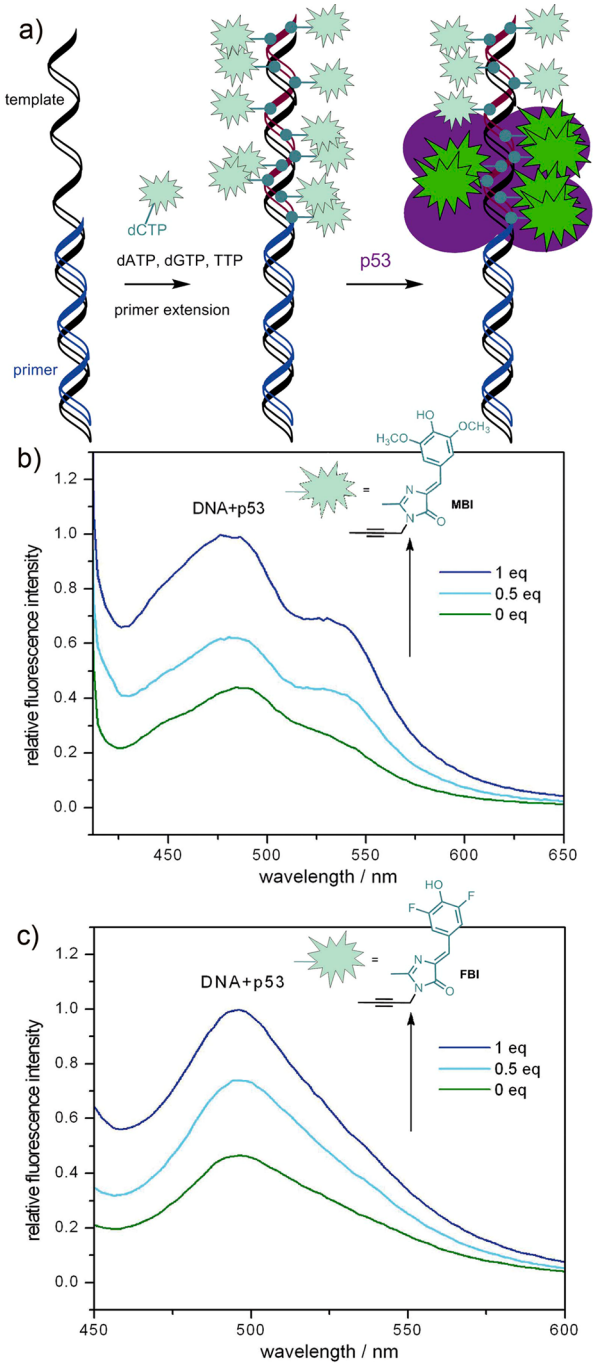

Figure 2. (a) Scheme of PEX incorporation of **dC^{MBI}TP** and **dC^{FBI}TP** to dsDNA and binding of p53. Photophysical properties of (b) **dC^{MBI}**- or (c) **dC^{FBI}**-labeled DNA upon binding of p53 protein.

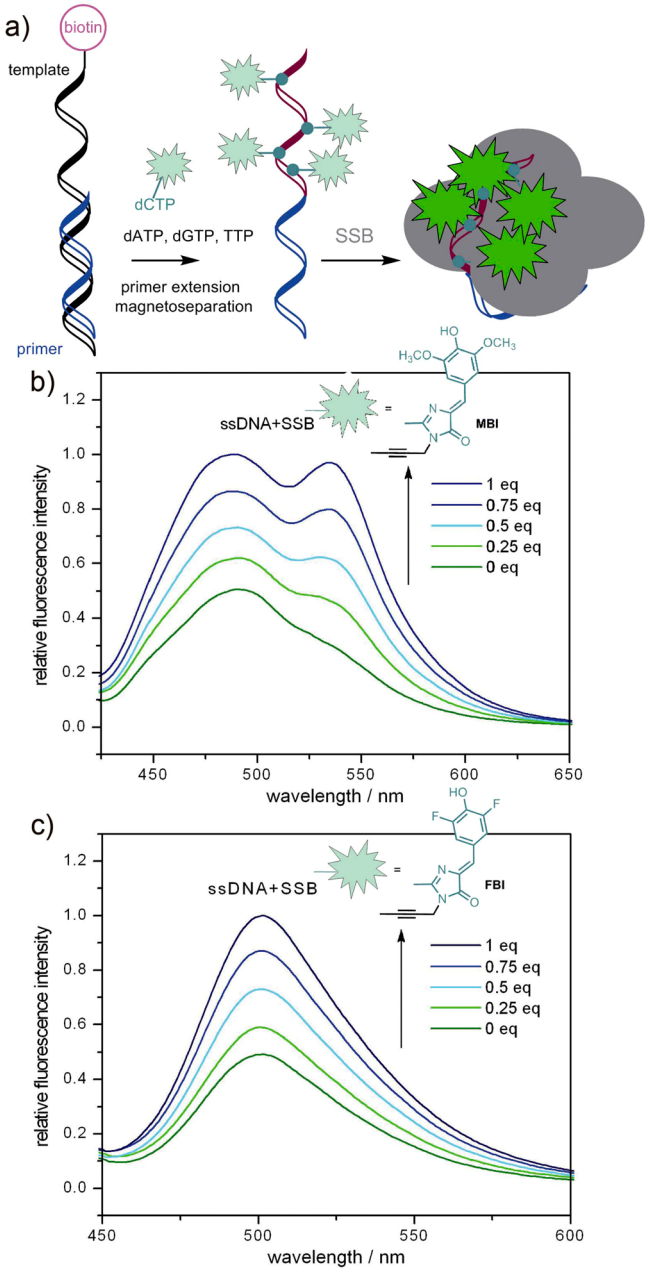

Figure 3. (a) Scheme of PEX incorporation of **dC^{MBI}TP** and **dC^{FBI}TP** to ssON and binding of SSB. Photophysical properties of (b) **dC^{MBI}**- or (c) **dC^{FBI}**-labeled ON upon binding of SSB protein.

of a further movement of the enzyme during primer extension (decrease of fluorescence). This approach using just a single modified dNTP is much simpler and more straightforward than standard FRET techniques for the studying of the primer extension. The concept certainly has a potential for studying of other enzymatic reactions, e.g., DNA repair of methylation. Studies along these lines will continue in our laboratories.

■ EXPERIMENTAL SECTION

NMR spectra were recorded on a 600 MHz (600.1 MHz for ^{1}H, 150.9 MHz for ^{13}C) or a 500 MHz (499.8 or 500.0 MHz for ^{1}H, 470.3 MHz for ^{19}F, 200.3 MHz for ^{31}P, 125.7 MHz for ^{13}C) spectrometer from sample solutions in D_2O, DMSO-d_6, or $CDCl_3$. Chemical shifts (in ppm, δ scale) were referenced as follows: $CDCl_3$ solutions, ^{1}H referenced to TMS (δ = 0 ppm), ^{13}C referenced to the solvent signal (δ = 77.0 ppm); DMSO-d_6 solutions, ^{1}H referenced to the residual solvent signal (δ = 2.50 ppm), ^{13}C referenced to the solvent signal (δ = 39.7 ppm); D_2O solutions, referenced to dioxane as an internal standard ($\delta(^{1}H)$ = 3.75 ppm, $\delta(^{13}C)$ = 69.3 ppm). ^{31}P NMR spectra were referenced to the phosphate buffer signal (δ = 2.35 ppm). ^{19}F NMR spectra were referenced to C_6F_6 as an external standard (δ = −163.0 ppm). Coupling constants (J) are given in Hz. NMR spectra of dNTPs were measured in phosphate buffer at pH 7.1. Complete assignment of all NMR signals was achieved by using a combination of H,H-COSY, H,C-HSQC, and H,C-HMBC experiments. Semipreparative separation of nucleoside triphosphates was performed by HPLC on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18 (2)). High resolution mass spectra were measured using ESI ionization technique and Orbitrap analyzer. Mass spectra of function-

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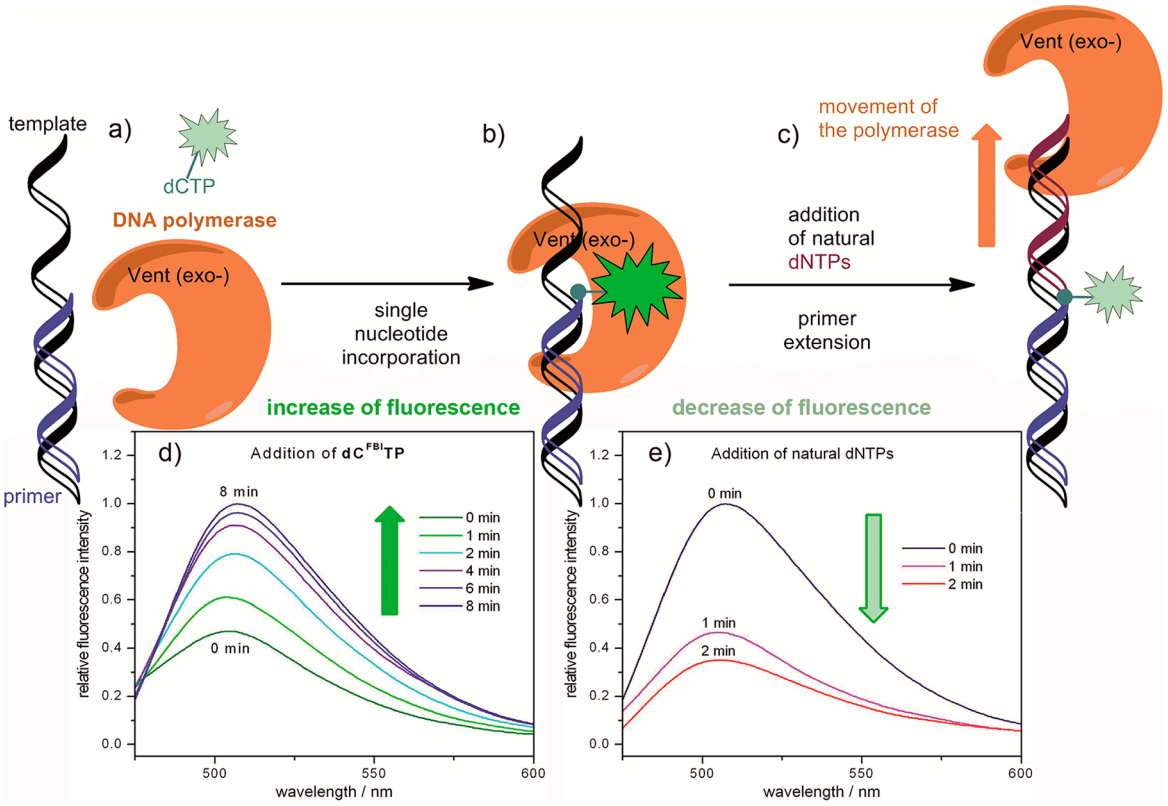

Figure 4. Scheme of time-resolved monitoring of primer extension. (a) Primer, template, **dC^{FBI}TP**, and Vent(exo-) are mixed, (b) single-nucleotide incorporation occurs, and (c) after the addition of a mixture of natural dNTPs, the PEX continues. Time-resolved fluorescence spectroscopy of (d) single-nucleotide incorporation **dC^{FBI}** to DNA, followed by (e) PEX after the addition of natural dNTPs.

alized DNA were measured using MALDI-TOF ionization with nitrogen laser.

General Procedure for the Synthesis of *N*-Propargyl-4-hydroxy-3,5-dimethoxybenzylidene Imidazolinones. Benzylidene oxazolinones[14] **1a** or **1b** were refluxed with 99% ethanol (7.5 or 15 mL), propargyl amine (1.5 equiv), and potassium carbonate (1.5 equiv) for 3 h. After cooling, the formed orange precipitate was filtered and washed by cold ethanol. The precipitate was redissolved in a 1:1 mixture of ethyl acetate and 500 mM sodium acetate (pH = 3.0). The organic layer was separated, and the solvent was removed under reduced pressure.

(*Z*)-4-(3,5-Dimethoxy-4-hydroxybenzylidene)-2-methyl-1-propargyl-imidazoline-5-one (P-MBI, 2a). **P-MBI** (**2a**) was prepared according to the general procedure from **1a** (1.12 g, 4.2 mmol, 1 equiv), propargylamine (0.34 g, 6.3 mmol, 1.5 equiv), and potassium carbonate (0.86 g, 6.3 mmol, 1.5 equiv). The product was isolated as an orange solid (601 mg, 47%). Mp 180−185 °C. ^{1}H NMR (499.8 MHz, DMSO-d_6): 2.42 (d, 3H, ^6J = 0.6, CH_3-2); 3.36 (t, 1H, ^4J = 2.5, HC≡C); 3.80 (s, 6H, CH_3O); 4.45 (d, 2H, ^4J = 2.5, CH_2N); 6.97 (s, 1H, CH=); 7.64 (s, 2H, H-*o*-$C_6H_2OH(OMe)_2$). ^{13}C NMR (125.7 MHz, DMSO-d_6): 15.7 (CH_3-2); 29.14 (CH_2N); 56.2 (CH_3O); 74.8 (HC≡C); 78.7 (C≡CH); 110.5 (CH-*o*-$C_6H_2OH(OMe)_2$); 124.5 (C-*i*-$C_6H_2OH(OMe)_2$); 127.5 (CH=); 135.8 (C-4-imid); 139.1 (C-*p*-$C_6H_2OH(OMe)_2$); 148.0 (C-*m*-$C_6H_2OH(OMe)_2$); 160.8 (C-2-imid); 168.8 (C-5-imid). MS (ESI$^+$): *m*/*z* (%) 301 (100) [M + H]$^+$, 323 (10) [M + Na]$^+$; HR-MS (ESI$^+$) for $C_{16}H_{16}O_4N_2Na$: [M + Na]$^+$ calculated 323.10023, found 323.10025.

(*Z*)-4-(3′,5′-Difluoro-4′-hydroxybenzylidene)-2-methyl-1-propargylimidazoline-5-one (P-FBI, 2b). **P-FBI** (**2b**) was prepared according to the general procedure from **1b** (500 mg, 2.09 mmol, 1 equiv), propargyl amine (0.17 g, 3.1 mmol, 1.5 equiv), and potassium carbonate (0.43 g, 3.1 mmol, 1.5 equiv). The separation required additional purification, using silica gel column chromatography (hexane/ethyl acetate 0−20% as eluent). The product was isolated as yellow solid (144 mg, 25%). Mp 204−207 °C. ^{1}H NMR (499.8 MHz, CD_3OD): 2.50 (d, 3H, ^6J = 0.6, CH_3-2 exchageable); 2.81 (t, 1H, ^4J = 2.5, HC≡); 4.46 (d, 2H, ^4J = 2.5, CH_2N); 6.92 (s, 1H, HC=); 7.79 (m, 2H, H-*o*-$C_6H_2F_2OH$). ^{13}C NMR (125.7 MHz, CD_3OD): 15.1 (CH_3-2); 30.1 (CH_2N); 73.9 (HC≡C−); 78.3 (-C≡CH); 116.4, 116.6 (2 × t, $J_{C,F}$ = 6.2, CH-*o*-$C_6H_2F_2OH$); 125.9 (t, $J_{C,F}$ = 9.3, C-*i*-$C_6H_2F_2OH$); 126.8 (=CH); 138.0 (t, $J_{C,F}$ = 16.6, C-*p*-$C_6H_2F_2OH$); 138.8 (C-4-imid); 153.5 (dd, $J_{C,F}$ = 242.2, 7.2, C-*m*-$C_6H_2F_2OH$); 163.8 (C-2-imid); 170.7 (C-5-imid). ^{19}F{^{1}H} NMR (470.3 MHz, CD_3OD): −131.79. MS (ESI$^+$): *m*/*z* (%) 277 (50) [M + H]$^+$, 299 (100) [M + Na]$^+$; HR-MS (ESI$^+$) for $C_{14}H_{11}O_2N_2F_2$: [M + H]$^+$ calculated 277.07831, found 277.07822.

General Procedure for Sonogashira Cross-Coupling of Base-Halogenated Nucleoside Triphosphates Analogues (dN^{I}TPs) with Propargyl MBI and FBI. A mixture of H_2O/CH_3CN (2:1, 2 mL) was added to an argon-purged flask containing **dC^{I}TP** (0.05 mmol), an acetylene **2a** or **2b** (0.075 mmol, 1.5 equiv), and CuI (0.95 mg, 0.005 mmol, 10 mol %). In a separate flask, $Pd(OAc)_2$ (0.56 mg, 0.0025 mmol, 5 mol %) and $P(Ph\text{-}SO_3Na)_3$ (3.59 mg, 0.00625 mmol, 2.5 equiv to Pd) were combined, evacuated, and purged with argon, followed by the addition of H_2O/CH_3CN (2:1, 0.5 mL). The mixture of the catalyst was then injected to the reaction mixture, *N*,*N*-diisopropylethylamine (43 μL, 0.25 mmol, 5 equiv) was added, and reaction mixture was stirred at 55 °C for 45 min. After cooling to room temperature, 200 μL of 0.5 M EDTA solution was added to the reaction mixture to remove the palladium catalyst. The products were isolated by C18 reverse phase column chromatogryphy using water−methanol (5−100%) containing 0.1 M TEAB buffer as eluent. The dNTPs were converted to sodium salt using ionex Dowex-50, evaporated and lyophilized.

5-{3′′′′-[(*Z*)-4′′′-(3′′′,5′′′-Dimethoxy-4′′′-hydroxybenzylidene)-2′′-methylimidazoline-5′′-one-1′′-*N*-yl]-1′′-propyn-1′′′′-yl}-2′-deoxycytidine-5′-*O*-triphosphate (dC^{MBI}TP, 3a). **dC^{MBI}TP** was prepared according to the general procedure, using **dC^{I}TP** (33 mg, 0.05 mmol, 1 equiv) and **2a** (22.5 mg, 0.075 mmol, 1.5 equiv). The product was isolated as yellow solid (13.4 mg, 21%). ^{1}H NMR

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(499.8 MHz, D_2O, pD = 7.1, phosphate buffer): 2.28 (dt, 1H, J_{gem} = 14.0, $J_{2'b,1'} = J_{2'b,3'}$ = 6.7, H-2′b); 2.42 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.3, $J_{2'a,3'}$ = 4.0, H-2′a); 2.51 (m, residual signal of exchengable CH_3-2); 3.89 (s, 6H, CH_3O); 4.20 (m, 3H, H-4′,5′); 4.58 (dt, 1H, $J_{3',2'}$ = 6.7, 4.0, $J_{3',4'}$ = 4.0, H-3′); 4.70 (s, 2H, CH_2N); 6.17 (dd, 1H, $J_{1',2'}$ = 6.7, 6.3, H-1′); 6.86 (s, 1H, CH═); 7.28 (s, 2H, H-*o*-$C_6H_2OH(OMe)_2$); 8.13 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer): 17.1 (CH_3-2); 33.6 (CH_2N); 41.9 (CH_2-2′); 58.8 (CH_3O); 68.0 (d, $J_{C,P}$ = 5.9, CH_2-5′); 73.1 (CH-3′); 77.2 (pyrimid-**C**≡C-imid); 88.2 (d, $J_{C,P}$ = 9.0, CH-4′); 89.2 (CH-1′); 91.8 (pyrimid-C≡**C**-imid); 94.2 (C-5); 112.7 (CH-*o*-$C_6H_2OH(OMe)_2$); 127.5 (C-*i*-$C_6H_2OH(OMe)_2$); 133.1 (CH═); 137.2 (C-4-imid); 140.7 (C-*p*-$C_6H_2OH(OMe)_2$); 148.1 (CH-6); 150.2 (C-*m*-$C_6H_2OH(OMe)_2$); 158.6 (C-2); 165.1 (C-2-imid); 167.7 (C-4); 173.4 (C-5-imid). $^{31}P\{^1H\}$ NMR (202.3 MHz, D_2O, pD = 7.1, phosphate buffer, ref(phosphate buffer) = 2.35 ppm): −21.34 (t, *J* = 20.0, P_β); −9.99 (d, *J* = 20.0, P_α); −7.74 (d, *J* = 20.0, P_γ). MS (ESI^-): *m*/*z* (%) 684.2 (30) $[M + 2H - PO_3H]^-$, 764.2 (100) $[M + 2H]^-$, 786.2 (30) $[M + H + Na]^-$; HR-MS (ESI^-) for $C_{25}H_{29}O_{17}N_5P_3$: $[M + 2H]^-$ calculated 764.07768, found 764.07615.

5-{3′′′′-[(*Z*)-4′′-(3′′′,5′′′-Difluoro-4′′′-hydroxybenzylidene)-2′′-methylimidazoline-5′′-one-1′′-*N*-yl]-1′′′′-propyn-1′′′′-yl}-2′-deoxycytidine-5′-*O*-triphosphate (dC^{FBI}TP, 3b). **dC^{FBI}TP** was prepared according to the general procedure, using **dC^{I}TP** (33 mg, 0.05 mmol, 1 equiv) and **2b** (20.7 mg, 0.075 mmol, 1.5 equiv). The product was isolated as yellow solid (11.5 mg, 28%). ^1H NMR (499.8 MHz, D_2O, pD = 7.1, phosphate buffer): 2.30 (dt, 1H, J_{gem} = 14.0, $J_{2'b,1'} = J_{2'b,3'}$ = 6.4, H-2′b); 2.43 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 6.4, $J_{2'a,3'}$ = 4.1, H-2′a); 2.50 (m, residual signal of exchengable CH_3-2); 4.20 (m, 3H, H-4′,5′); 4.59 (dt, 1H, $J_{3',2'}$ = 6.4, 4.1, $J_{3',4'}$ = 4.1, H-3′); 4.72 (s, 2H, CH_2N); 6.20 (t, 1H, $J_{1',2'}$ = 6.4, H-1′); 7.04 (s, 1H, CH═); 7.57 (m, 2H, H-*o*-$C_6H_2F_2OH$); 8.14 (s, 1H, H-6). ^{13}C NMR (125.7 MHz, D_2O, pD = 7.1, phosphate buffer): 17.3 (CH_3-2); 33.6 (CH_2N); 41.8 (CH_2-2′); 67.9 (d, $J_{C,P}$ = 5.5, CH_2-5′); 73.0 (CH-3′); 77.1 (pyrimid-**C**≡C-imid); 88.2 (d, $J_{C,P}$ = 8.8, CH-4′); 89.2 (CH-1′); 92.0 (pyrimid-C≡**C**-imid); 94.3 (C-5); 118.41, 118.6 (2 × t, $J_{C,F}$ = 6.6, CH-*o*-$C_6H_2F_2OH$); 119.0 (t, $J_{C,F}$ = 9.8, C-*i*-$C_6H_2F_2OH$); 133.7 (CH═); 135.5 (C-4-imid); 148.1 (CH-6); 151.8 (br, C-*p*-$C_6H_2F_2OH$); 157.9 (dd, $J_{C,F}$ = 237.9, 10.7, C-*m*-$C_6H_2F_2OH$); 158.7 (C-2); 163.6 (C-2-imid); 167.75 (C-4); 173.5 (C-5-imid). $^{31}P\{^1H\}$ NMR (202.3 MHz, D_2O, pD = 7.1, phosphate buffer, ref(phosphate buffer) = 2.35 ppm): −21.30 (t, *J* = 19.6, P_β); −10.26 (d, *J* = 19.6, P_α); −6.88 (d, *J* = 19.6, P_γ). $^{19}F\{^1H\}$ NMR (470.3 MHz, D_2O, pD = 7.1, phosphate buffer): −131.70. MS (ESI^-): *m*/*z* (%) 581.2 (20) $[M + 2H-2PO_3H]^-$, 603.2 (40) $[M + H + Na - 2PO_3H]^-$ 660.2 (8) $[M + 2H - PO_3H]^-$, 740.1 (3) $[M + 2H]^-$; HR-MS (ESI^-) for $C_{23}H_{23}O_{15}N_5F_2P_3$: $[M + 2H]^-$ calculated 740.03770, found 740.03726.

Binding Study Using SSB Protein. SSB Protein from *E. coli* was purchased from Sigma-Aldrich and concentrated 6x using Amicon Ultra 0.5 Centrifugal Filter Unit (cutoff 3KDa). The measurements were performed in 20 mM phosphate buffer pH 7.4 using three parallel samples for exact comparison (DNA mixed with binding protein, DNA diluted with buffer, and control sample − DNA mixed with BSA and glycerol). An appropriate amount of glycerol was added to negative control to refine the data from the effect of glycerol present in the stock solution of SSB protein. BSA was used as control protein. The measurements were performed with an AMINCO Bowman series 2 spectrofluorometer.

Binding Study Using p53 Protein. Wild type human full length p53 proteins were expressed in *E. coli* BL21/DE3 and purified as described previously.[22] The measurements were performed in 50 mM KCl, 5 mM Tris pH 7.6, 2 mM DTT, 0.01% Triton-X100, using three parallel samples for exact comparison (DNA mixed with binding protein, DNA diluted with buffer, and control sample − DNA mixed with BSA). BSA was used as control protein. The measurements were performed using a PC-1 steady-state ISS spectrofluorometer.

Binding Study Using Vent(exo-) DNA Polymerase. Vent(exo-) (2 U/μL, 0.5 μM, 500 μL) was diluted with water (200 μL) and concentrated on an Amicon Ultra Centrifugal Filter Unit (cutoff 3 kDa, Millipore) to the final volume of 75 μL. Template (temp^{vent} 5′-CTAGCATGAGCTCAGGCCCATGCCGCCCATG-3′, 100 μM, 2.4 μL), primer (prim^{vent} 5′-CATGGGCGGCATGGGC-3′, 100 μM, 2.4 μL), **dC^{FBI}TP** (40 μM, 7.8 μL), ThermoPol reaction buffer (10×, 12 μL) and water (20.4 μL) were mixed. Next, the preconcentrated solution of Vent(exo-) was added, and the fluorescence was measured immediately and then after every minute until the fluorescence stopped increasing (after 8 min). Then a mixture of natural dNTPs (4 mM, 6 μL) was added to the reaction mixture, and the fluorescence was measured every minute until the change in fluorescence intensity was completed (2 min).

Primer Extension. Primer Extension for Analysis by Polyacrylamide Gel Electrophoresis. The reaction mixture (20 μL) contained primer (0.15 μM), template (0.22 μM), natural dNTPs (200 μM), dN^{R}TPs, buffer, and DNA polymerase (0.1 U). The reaction mixture was incubated for 15 min (30 min for quadruple-modified ONs) at 60 °C and analyzed by polyacrylamide gel electrophoresis. The primers were ^{32}P-prelabeled at 5′-end to allow radiographic detection.

Preparative Primer Extension for Fluorescence Studies. Reaction mixture (500 μL) for the oligonucleotide preparation contained primer (6.6 μM), 5′-biotinylated template (6.6 μM), dNTPs (200 μM), dN^{R}TPs (200 μM), 10x buffer, and KOD XL DNA polymerase (7 U). The reaction was incubated for 40 min. Biotinylated templates were used to allow magnetoseparation.

Isolation and Characterization of Single-Strand Oligonucleotides by Magnetoseparatic Procedure. MagPrep P-25 (50 μL, Streptavidin Particles stock solution from Novagen) was washed three times with 450 μL of buffer (0.3 M NaCl, 10 mM TRIS, pH = 7.4). The reaction mixture containing 0.3 M NaCl was added to a suspension of magnetic beads. The suspension was shaken for 40 min at room temperature, allowing binding of oligonucleotides to MagPrep beads. The beads were washed three times with 500 μL of PBS solution (0.14 M NaCl, 3 mM KCl, 4 mM sodium phosphate pH = 7.4), three times with 500 μL of TRIS buffer (0.3 M NaCl, 10 mM TRIS, pH = 7.4), and three times with 500 μL of deionized water. Single-stranded oligonucleotides were released by shaking and heating the sample to 60 °C for 2 min, followed by shaking with additional magnetic beads in Tris buffer to remove small amounts of biotinylated template released during heating at 60 °C in deionized water. Each medium exchange was performed using a magnetoseparator (Dynal, Norway). The oligonucleotides were desalted and concentrated using Amicon Ultra 0.5 Centrifugal Filter Unit (cutoff 3 kDa). Characterization of oligonucleotides by MS (MALDI-TOF): $[M + H]^+$ pex^{rnd16}(**dC^{MBI}**) calcd 10810.5, found 10810.2; pex^{rnd16}(**dC^{FBI}**) calcd 10715.1, found 10716.6.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures of fluorescence and UV−vis spectra and copies of all NMR spectra and MALDI-TOF spectra. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to the memory of Dr. Detlef Schröder.

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