Four-color DNA sequencing by synthesis on a chip using photocleavable fluorescent nucleotides

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We report four-color DNA sequencing by synthesis (SBS) on a chip, using four photocleavable fluorescent nucleotide analogues (dGTP-PC-Bodipy-FL-510, dUTP-PC-R6G, dATP-PC-ROX, and dCTP-PC-Bodipy-650) (PC, photocleavable; Bodipy, 4,4-difluoro-4-bora-3α,4α-diaza-*s*-indacene; ROX, 6-carboxy-X-rhodamine; R6G, 6-carboxyrhodamine-6G). Each nucleotide analogue consists of a different fluorophore attached to the 5 position of the pyrimidines and the 7 position of the purines through a photocleavable 2-nitrobenzyl linker. After verifying that these nucleotides could be successfully incorporated into a growing DNA strand in a solution-phase polymerase reaction and the fluorophore could be cleaved using laser irradiation (≈355 nm) in 10 sec, we then performed an SBS reaction on a chip that contains a self-priming DNA template covalently immobilized by using 1,3-dipolar azide-alkyne cycloaddition. The DNA template was produced by PCR, using an azido-labeled primer, and the self-priming moiety was attached to the immobilized DNA template by enzymatic ligation. Each cycle of SBS consists of the incorporation of the photocleavable fluorescent nucleotide into the DNA, detection of the fluorescent signal, and photocleavage of the fluorophore. The entire process was repeated to identify 12 continuous bases in the DNA template. These results demonstrate that photocleavable fluorescent nucleotide analogues can be incorporated accurately into a growing DNA strand during a polymerase reaction in solution and on a chip. Moreover, all four fluorophores can be detected and then efficiently cleaved using near-UV irradiation, thereby allowing continuous identification of the DNA template sequence. Optimization of the steps involved in this SBS approach will further increase the read-length.

2-nitrobenzyl linker | DNA chip | photocleavage

DNA sequencing is a fundamental tool for biological science. The completion of the Human Genome Project has set the stage for screening genetic mutations to identify disease genes on a genome-wide scale (1). Accurate high-throughput DNA sequencing methods are needed to explore the complete human genome sequence for applications in clinical medicine and health care. Recent studies have indicated that an important route for identifying functional elements in the human genome involves sequencing the genomes of many species representing a wide sampling of the evolutionary tree (2). To overcome the limitations of the current electrophoresis-based sequencing technology (3–5), a variety of new DNA sequencing methods have been investigated. Such approaches include sequencing by hybridization (6), MS-based sequencing (7–9), and sequence-specific detection of single-stranded DNA by using engineered nanopores (10). More recently, DNA sequencing by synthesis (SBS) approaches such as pyrosequencing (11), sequencing of single DNA molecules (12), and polymerase colonies (13) have been widely explored.

The concept of DNA SBS was revealed in 1988 (14). This approach involves detection of the identity of each nucleotide immediately after its incorporation into a growing strand of DNA in a polymerase reaction. Thus far, no complete success has been reported in using such a system to sequence DNA unambiguously. We proposed an SBS approach using photocleavable fluorescent nucleotide analogues on a surface in 2000 (15). In this approach, modified nucleotides are used as reversible terminators, in which a different fluorophore with a distinct fluorescent emission is linked to each of the four bases through a photocleavable linker, and the 3′-OH group is capped by a small chemical moiety. DNA polymerase incorporates only a single-nucleotide analogue complementary to the base on a DNA template covalently linked to a surface. After incorporation, the unique fluorescence emission is detected to identify the incorporated nucleotide, and the fluorophore is subsequently removed photochemically. The 3′-OH group is then chemically regenerated, allowing the next cycle of the polymerase reaction to proceed. Because the large surface on a DNA chip can have a high density of different DNA templates spotted, each cycle can identify many bases in parallel, allowing the simultaneous sequencing of a large number of DNA molecules. The advantage of using photons as reagents for initiating photoreactions to cleave the fluorophore is that no additional chemical reagents are required to be introduced into the system, and clean products can be generated with no need for subsequent purification. We have previously established the feasibility of performing SBS on a chip using a synthetic DNA template and photocleavable pyrimidine nucleotides (C and U) (16). As further development of this approach, we report here the design and synthesis of four photocleavable nucleotide analogues (A, C, G, and U), each of which contains a unique fluorophore with a distinct fluorescence emission. We first established that these nucleotides are good substrates for DNA polymerase in a solution-phase DNA extension reaction and that the fluorophore can be removed with high speed and efficiency by laser irradiation (≈355 nm). We then performed SBS using these four photocleavable nucleotide analogues to identify the sequence of a DNA template immobilized on a chip. The DNA template was produced by PCR, using an azido-labeled primer, and was immobilized on the surface of the chip with 1,3-dipolar azide-alkyne cycloaddition chemistry. A self-priming moiety was then covalently attached to the DNA template by enzymatic ligation to allow the polymerase reaction to proceed on the DNA immobilized on the surface. To the best of our knowledge, this is the first report of using a complete set of photocleavable fluorescent nucleotides for four-color DNA SBS. In an accompanying paper (17), we report the design and synthesis of a photocleavable fluorescent nucleotide as a reversible terminator for SBS.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. ^{1}H NMR spectra were recorded on a Bruker

Abbreviations: Bodipy, 4,4-difluoro-4-bora-3α,4α-diaza-*s*-indacene; PC, photocleavable; R6G, 6-carboxyrhodamine-6G; ROX, 6-carboxy-X-rhodamine; SBS, sequencing by synthesis; YAG, yttrium/aluminum garnet.

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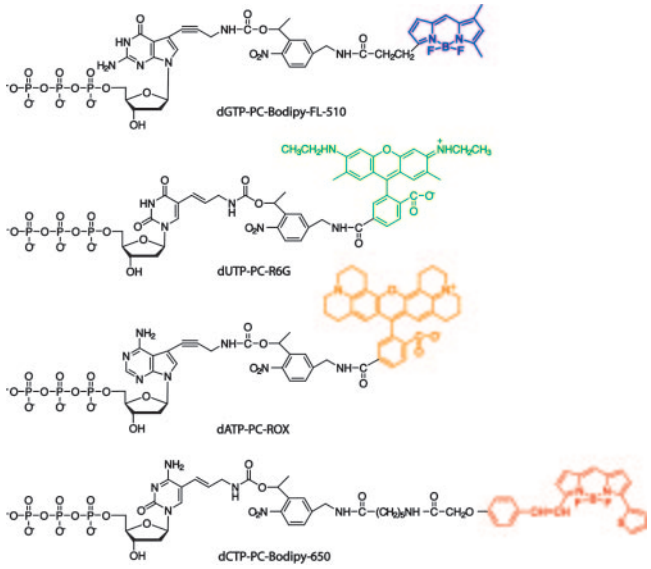


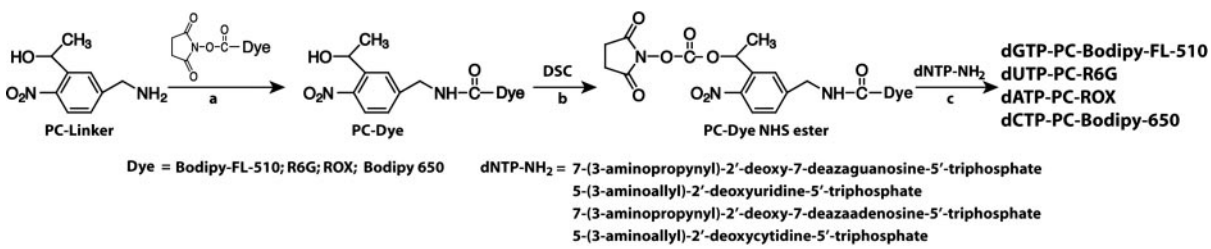
Fig. 1. Structures of dGTP-PC-Bodipy-FL-510 [$\lambda_{abs\ (max)}$ = 502 nm; $\lambda_{em\ (max)}$ = 510 nm], dUTP-PC-R6G [$\lambda_{abs\ (max)}$ = 525 nm; $\lambda_{em\ (max)}$ = 550 nm], dATP-PC-ROX [$\lambda_{abs\ (max)}$ = 575 nm; $\lambda_{em\ (max)}$ = 602 nm], and dCTP-PC-Bodipy-650 [$\lambda_{abs\ (max)}$ = 630 nm; $\lambda_{em\ (max)}$ = 650 nm].

400 spectrometer. High-resolution MS data were obtained by using a JEOL JMS HX 110A mass spectrometer. Mass measurement of DNA was made on a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems). Photolysis was performed by using a Spectra-Physics GCR-150-30 Nd-yttrium/aluminum garnet (YAG) laser that generates light pulses at 355 nm (*ca.* 50 mJ/pulse, pulse length ≈7 ns) at a frequency of 30 Hz with a light intensity at ≈1.5 W/cm^2. The scanned fluorescence emission images were obtained by using a ScanArray Express scanner (PerkinElmer Life Sciences) equipped with four lasers with excitation wavelengths of 488, 543, 594, and 633 nm and emission filters centered at 522, 570, 614, and 670 nm.

Synthesis of Photocleavable Fluorescent Nucleotides. Photocleavable fluorescent nucleotides dGTP-PC-Bodipy-FL-510, dUTP-PC-R6G, dATP-PC-ROX, and dCTP-PC-Bodipy-650 (PC, photocleavable; Bodipy, 4,4-difluoro-4-bora-3α,4α-diaza-*s*-indacene; ROX, 6-carboxy-X-rhodamine; R6G, 6-carboxyrhodamine-6G) (Fig. 1) were synthesized according to Scheme 1, using a similar method to that reported in ref. 16. A photocleavable linker (PC-Linker) 1-[5-(aminomethyl)-2-nitrophenyl]ethanol was reacted with the *N*-hydroxysuccinimide (NHS) ester of the corresponding fluorescent dye to produce an intermediate PC-Dye, which was converted to a PC-Dye NHS ester by reacting with *N*,*N*′-disuccinimidyl carbonate. The coupling reaction between the different PC-Dye NHS esters and the amino nucleotides (dATP-NH_2 and dGTP-NH_2 from PerkinElmer, dUTP-NH_2 from Sigma, and dCTP-NH_2 from TriLink BioTechnologies) produced the four photocleavable fluorescent nucleotides. Detailed synthesis procedures and all characterization data for newly synthesized molecules are reported in the supporting information, which is published on the PNAS web site.

DNA Polymerase Reaction Using Four Photocleavable Fluorescent Nucleotide Analogues in Solution. We characterized the four nucleotide analogues, dGTP-PC-Bodipy-FL-510, dUTP-PC-R6G, dATP-PC-ROX, and dCTP-PC-Bodipy-650, by performing four continuous DNA extension reactions sequentially using a primer (5′-AGAGGATCCAACCGAGAC-3′) and a synthetic 60-mer DNA template (5′-GTGTACATCAACATCACCTACCAC-CATGTCAGTCTCGGTTGGAT-CCTCTATTGTGTCCGG-3′) corresponding to a portion of exon 7 of the human *p53* gene (Fig. 2). The four nucleotides in the template immediately adjacent to the annealing site of the primer were 3′-ACTG-5′. First, a polymerase extension reaction using dUTP-PC-R6G along with the primer and the template was performed producing a single-base extension product. The reaction mixture for this and all subsequent extension reactions consisted of 80 pmol of template, 50 pmol of primer, 80 pmol of the particular photocleavable fluorescent nucleotide, 1× Thermo Sequenase reaction buffer, and 4 units of Thermo Sequenase DNA polymerase (Amersham Pharmacia Biosciences) in a total volume of 20 μl. The reaction consisted of 25 cycles at 94°C for 20 sec, 48°C for 40 sec, and 60°C for 75 sec. Subsequently, the extension product was purified by using reverse-phase HPLC. An Xterra MS C18 (4.6 × 50-mm) column (Waters) was used for the HPLC purification. Elution was performed over 120 min at a flow rate of 0.5 ml/min with the temperature set at 50°C by using a linear gradient (12–34.5%) of methanol in a buffer consisting of 8.6 mM triethylamine and 100 mM hexafluoroisopropyl alcohol (pH 8.1). The fraction containing the desired DNA product was collected and freeze-dried for analysis by using MALDI-TOF MS. For photocleavage, the purified DNA extension product bearing the fluorescent nucleotide analogue was resuspended in 200 μl of deionized water. The mixture was irradiated for 10 seconds in a quartz cell with path lengths of 1.0 cm employing a Nd-YAG laser at 355 nm and then analyzed by MALDI-TOF MS. After photocleavage, the DNA product with the fluorophore removed was used as a primer for a second extension reaction with dGTP-PC-Bodipy-FL-510. The second extended product was then purified by HPLC and photolyzed. The third extension, using dATP-PC-ROX, and the fourth extension, using dCTP-PC-Bodipy-650, were carried out in a similar manner by using the previously extended and photocleaved product as the primer.

PCR Amplification to Produce Azido-Labeled DNA Template. An azido-labeled PCR product was obtained by using a 100-bp template (5′-AGCGACTGCTATCATGTCATATCGACGT-GCTCACTAGCTCTACATATGCGTGCGTGATCAGAT-GACGTATCGATGCTGACTATAGTCTCCCATGCGAG-



Scheme 1. Synthesis of photocleavable fluorescent nucleotides. a, acetonitrile or DMF/1 M $NaHCO_3$ solution; b, *N*,*N*′-disuccinimidyl carbonate (DSC), triethylamine; c, 0.1 M $Na_2CO_3/NaHCO_3$ aqueous buffer (pH 8.5–8.7).

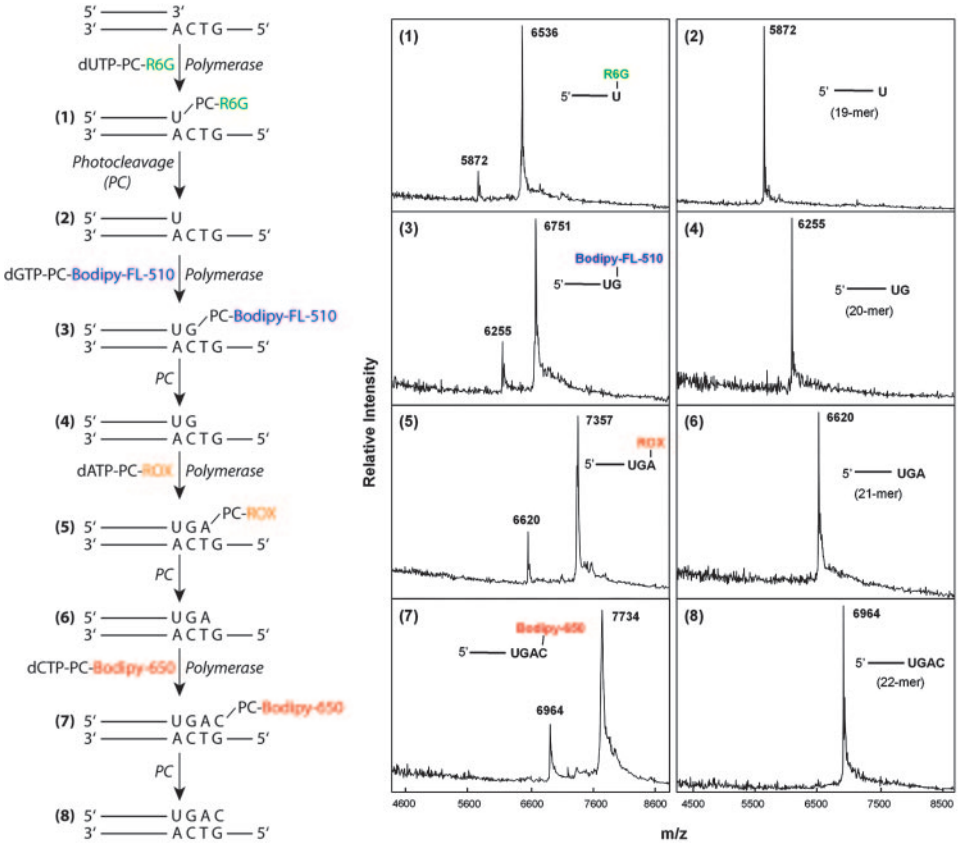
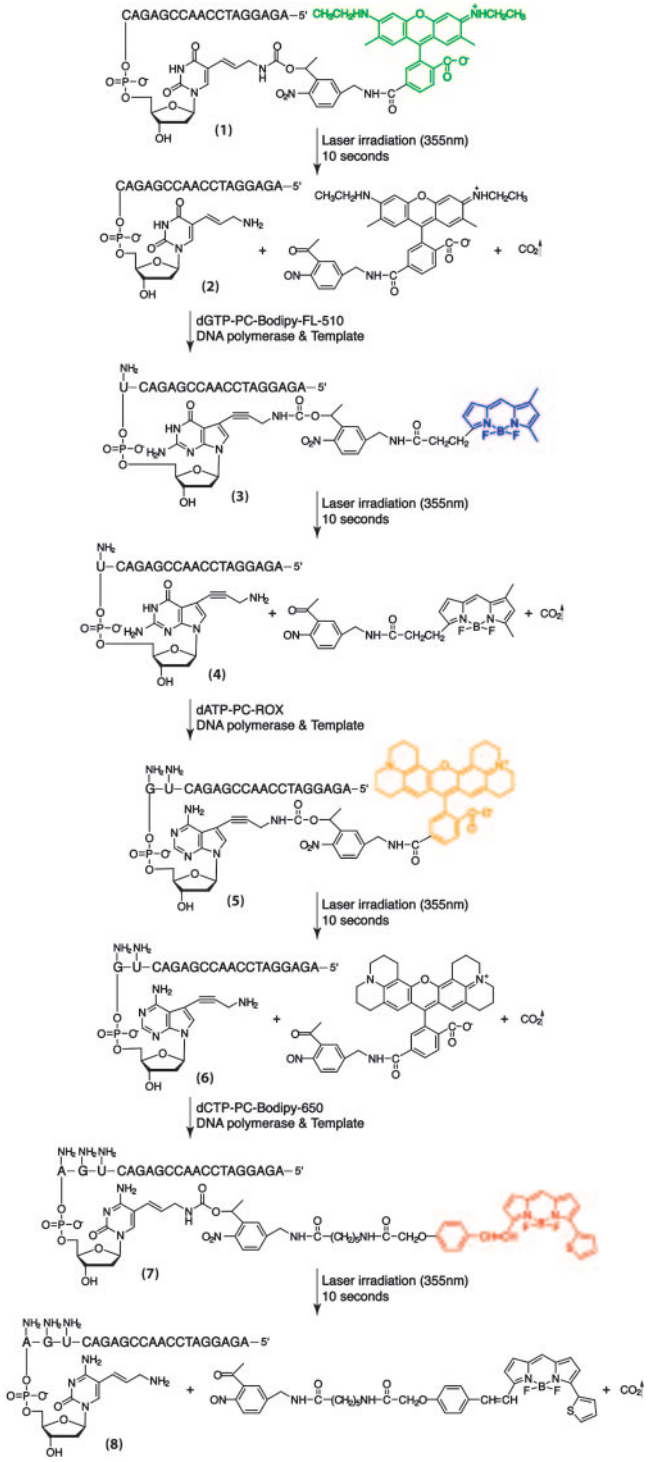


Fig. 2. The polymerase extension scheme (*Left*) and MALDI-TOF MS spectra of the four consecutive extension products and their photocleavage products (*Right*). Primer extended with dUTP-PC-R6G (**1**) and its photocleavage product **2**; product **2** extended with dGTP-PC-Bodipy-FL-510 (**3**), and its photocleavage product **4**; product **4** extended with dATP-PC-ROX (**5**), and its photocleavage product **6**; product **6** extended with dCTP-PC-Bodipy-650 (**7**), and its photocleavage product **8**. After 10 sec of irradiation with a laser at 355 nm, photocleavage is complete with all of the fluorophores cleaved from the extended DNA products.

TG-3′), a 24-bp azido-labeled forward primer (5′-N_3-AGCGACTGCTATCATGTCATATCG-3′), and a 24-bp unlabeled reverse primer (5′-CACTCGCATGGGAGACTATAGTCA-3′). In a total reaction volume of 50 μl, 1 pmol of template and 30 pmol of forward and reverse primers were mixed with 1 unit of AccuPrime *Pfx* DNA polymerase and 5 μl of 10× AccuPrime *Pfx* reaction mix (Invitrogen) containing 1 mM $MgSO_4$ and 0.3 mM dNTP. The PCR reaction consisted of an initial denaturation step at 95°C for 1 min, followed by 38 cycles at 94°C for 15 sec, 63°C for 30 sec, and 68°C for 30 sec. The product was purified by using a 96 QIAquick multiwell PCR purification kit (Qiagen), and the quality was checked by using 2% agarose gel electrophoresis in 1× TAE buffer. The concentration of the purified PCR product was measured by using a PerkinElmer Lambda 40 UV-vis spectrophotometer.

Construction of a Self-Priming DNA Template on a Chip by Enzymatic Ligation. The amino-modified glass slide (Sigma) was functionalized to contain a terminal alkynyl group as described in ref. 16. The azido-labeled DNA product generated by PCR was dissolved in DMSO/H_2O (1/3 vol/vol) to obtain a 20 μM solution. Five microliters of the DNA solution was mixed with CuI (10 nmol, 100 eq) and *N*,*N*-diisopropyl-ethylamine (10 nmol, 100 eq) and then spotted onto the alkynyl-modified glass surface in the form of 6-μl drops. The glass slide was incubated in a humid chamber at room temperature for 24 h, washed with deionized water (dH_2O) and SPSC buffer (50 mM sodium phosphate/1 M NaCl, pH 6.5) for 1 h (16), and finally rinsed with dH_2O. To denature the double-stranded PCR-amplified DNA to remove the non-azido-labeled strand, the glass slide was immersed into 0.1 M NaOH solution for 10 min and then washed with 0.1 M NaOH and dH_2O, producing a single-stranded DNA template that is immobilized on the chip. For the enzymatic ligation of a self-priming moiety to the immobilized DNA template on the chip, a 5′-phosphorylated 40-bp loop primer (5′-PO_3-GCTGAATTCCGCGTTCGCGGAATTCAGCCACTCGCATGGG-3′) was synthesized. This primer contained a thermally stable loop sequence, 3′-G(CTTG)C-5′, a 12-bp stem, and a 12-bp overhanging end that would be annealed to the immobilized single-stranded template at its 3′-end. A 10-μl solution consisting of 100 pmol of the primer, 10 units of *Taq*DNA ligase, 0.1 mM NAD, and 1× reaction buffer (New England Biolabs) was spotted onto a location of the chip containing the immobilized DNA and incubated at 45°C for 4 h. The glass slide was washed with dH_2O, SPSC buffer, and again with dH_2O. The formation of a stable hairpin was ascertained by covering the entire surface with 1× reaction buffer (26 mM Tris·HCl/6.5 mM $MgCl_2$, pH 9.3), incubating it in a humid chamber at 94°C for 5 min to dissociate any partial hairpin structure, and then slowly allowing it to cool down to room temperature for reannealing.

SBS Reaction on a Chip with Four Photocleavable Fluorescent Nucleotide Analogues. One microliter of a solution consisting of dATP-PC-ROX (60 pmol), 2 units of Thermo Sequenase DNA polymerase, and 1× reaction buffer was spotted on the surface of the chip, where the self-primed DNA moiety was immobilized. The nucleotide analogue was allowed to incorporate into the primer at 72°C for 5 min. After washing with a mixture of SPSC buffer, 0.1% SDS, and 0.1% Tween 20 for 10 min, the surface was rinsed with dH_2O and ethanol successively and then scanned with a



Scheme 2. DNA extension reaction performed in solution phase to characterize the four different photocleavable fluorescent nucleotide analogues (dUTP-PC-R6G, dGTP-PC-Bodipy-FL-510, dATP-PC-ROX, and dCTP-PC-Bodipy-650). After each extension reaction, the DNA extension product is purified by HPLC for MALDI-TOF MS measurement to verify that it is the correct extension product. Photolysis is performed to produce a DNA product that is used as a primer for the next DNA extension reaction.

ScanArray Express (PerkinElmer) scanner to detect the fluorescence signal. To perform photocleavage, the glass chip was placed inside a chamber (50 × 50 × 50 mm) filled with acetonitrile/water (1/1 vol/vol) solution and irradiated for 1 min

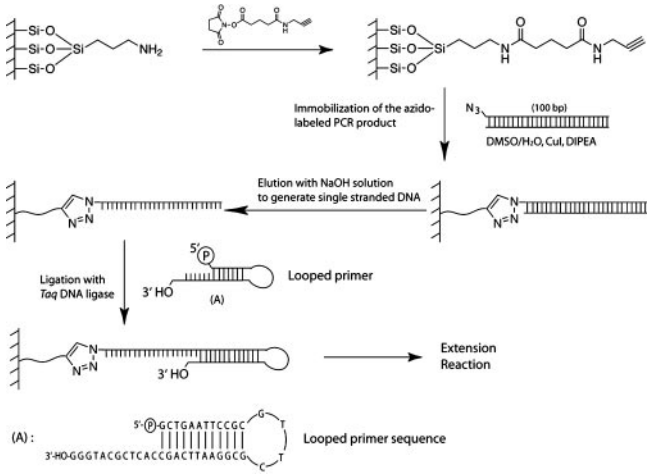


Fig. 3. Immobilization of an azido-labeled PCR product on an alkynyl-functionalized surface, and a ligation reaction between the immobilized single-stranded DNA template and a loop primer to form a self-priming DNA moiety on the chip. The sequence of the loop primer is shown in (A).

with the Nd-YAG laser at 355 nm. The light intensity applied on the glass surface was ≈1.5 W/cm^2. After washing the surface with dH_2O and ethanol, the surface was scanned again to compare the intensity of fluorescence after photocleavage with the original fluorescence intensity. This process was followed by the incorporation of dGTP-PC-Bodipy-FL-510, with the subsequent washing, fluorescence detection, and photocleavage processes performed as described above. The same cycle was repeated 10 more times with each of the four photocleavable fluorescent nucleotide analogues complementary to the base on the template. For a negative control experiment, a 1-μl solution containing dATP-PC-ROX (60 pmol) and 1× reaction buffer was added on to the DNA immobilized on the chip in the absence of DNA polymerase and then incubated at 72°C for 5 min, followed by the same washing and detection steps as above.

Results and Discussion

To demonstrate the feasibility of carrying out DNA SBS on a chip, four photocleavable fluorescent nucleotide analogues (dGTP-PC-Bodipy-FL-510, dUTP-PC-R6G, dATP-PC-ROX, and dCTP-PC-Bodipy-650) (Fig. 1) were synthesized according to Scheme 1, using a similar procedure as that reported in ref. 16. Modified DNA polymerases have been shown to be highly tolerant to nucleotide modifications with bulky groups at the 5 position of pyrimidines (C and U) and the 7 position of purines (A and G) (18, 19). Thus, we attached each unique fluorophore to the 5 position of C/U and the 7 position of A/G through a photocleavable 2-nitrobenzyl linker.

To verify that these fluorescent nucleotides are incorporated accurately in a base-specific manner in a polymerase reaction, four continuous steps of DNA extension and photocleavage by near-UV irradiation were carried out in solution as shown in Scheme 2. This allows the isolation of the DNA product at each step for detailed molecular structure characterization as shown in Fig. 2. The first extension product, 5′—U(PC-R6G)-3′ **1**, was purified by HPLC and analyzed by using MALDI-TOF MS (Fig. 2, panels 1). This product was then irradiated at 355 nm by using an Nd-YAG laser for 10 sec, and the photocleavage product **2** was also analyzed by using MALDI-TOF MS (Fig. 2, panels 2). Near-UV light absorption by the aromatic 2-nitrobenzyl linker causes reduction of the 2-nitro group to a nitroso group and an oxygen insertion into the carbon-hydrogen bond followed by cleavage and decarboxylation (20). As can be seen from Fig. 2, panels 1, the MALDI-TOF MS spectrum consists of a distinct

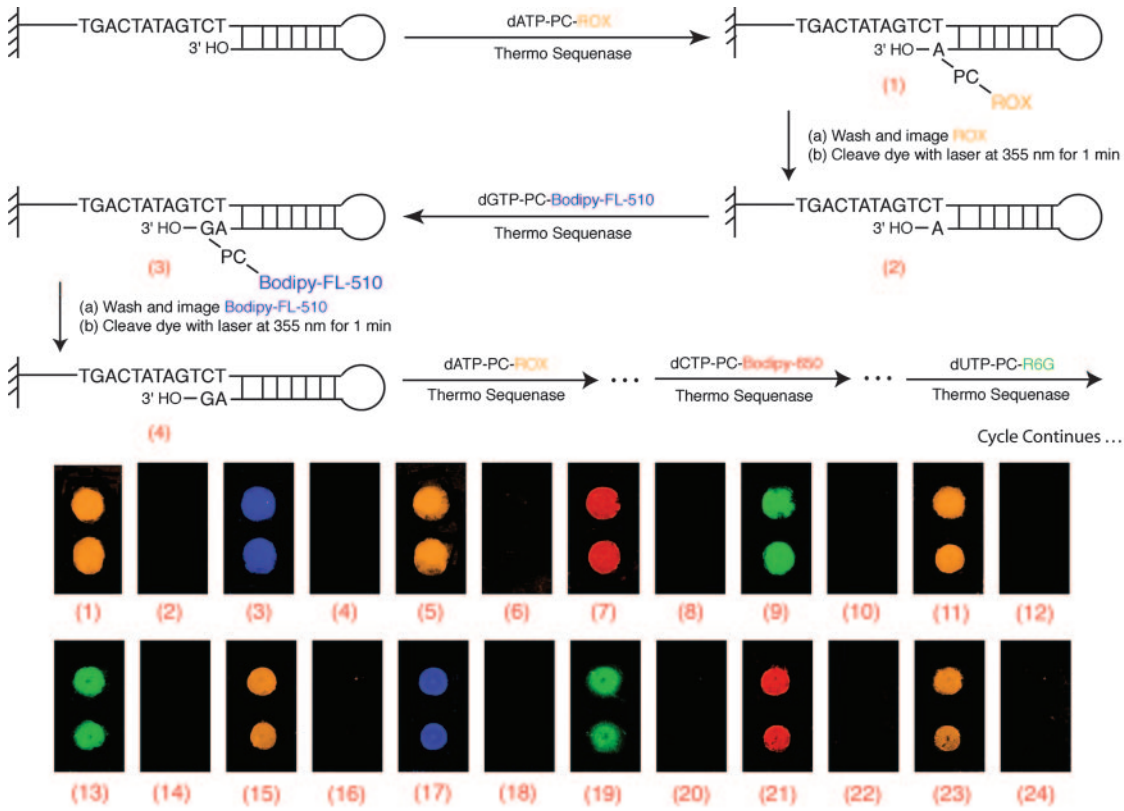


Fig. 4. Schematic representation of SBS on a chip using four PC fluorescent nucleotides (*Upper*), and the scanned fluorescence images for each step of SBS on a chip (*Lower*). 1, Incorporation of dATP-PC-ROX; 2, photocleavage of PC-ROX; 3, incorporation of dGTP-PC-Bodipy-FL-510; 4, photocleavage of PC-Bodipy-FL-510; 5, incorporation of dATP-PC-ROX; 6, photocleavage of PC-ROX; 7, incorporation of dCTP-PC-Bodipy-650; 8, photocleavage of PC-Bodipy-650; 9, incorporation of dUTP-PC-R6G; 10, photocleavage of PC-R6G; 11, incorporation of dATP-PC-ROX; 12, photocleavage of PC-ROX; 13, incorporation of dUTP-PC-R6G; 14, photocleavage of PC-R6G; 15, incorporation of dATP-PC-ROX; 16, photocleavage of PC-ROX; 17, incorporation of dGTP-PC-Bodipy-FL-510; 18, photocleavage of PC-Bodipy-FL-510; 19, incorporation of dUTP-PC-R6G; 20, photocleavage of PC-R6G; 21, incorporation of dCTP-PC-Bodipy-650; 22, photocleavage of PC-Bodipy-650; 23, incorporation of dATP-PC-ROX; 24, photocleavage of PC-ROX.

peak at m/z 6,536 corresponding to the DNA extension product 5′—U(PC-R6G)-3′ (**1**), confirming that the nucleotide analogue can be incorporated base-specifically by DNA polymerase into a growing DNA strand. The small peak at m/z 5,872 corresponding to the photocleavage product is due to the partial cleavage caused by the nitrogen laser pulse (337 nm) used in MALDI ionization. For photocleavage, a Nd-YAG laser was used to irradiate the DNA product carrying the fluorescent nucleotide for 10 sec at 355 nm to cleave the fluorophore from the DNA extension product. Fig. 2 (panels 2) shows the photocleavage result of the above DNA product. The peak at m/z 6,536 has completely disappeared, whereas the peak corresponding to the photocleavage product 5′—U (**2**) appears as the sole dominant peak at m/z 5,872, which establishes that laser irradiation completely cleaves the fluorophore with high speed and efficiency without damaging the DNA. The next extension reaction was carried out by using this photocleaved DNA product as a primer along with dGTP-PC-Bodipy-FL-510 to yield an extension product 5′—UG(PC-Bodipy-FL-510)-3′ (**3**). As described above, the extension product **3** was purified, analyzed by MALDI-TOF MS producing a dominant peak at m/z 6,751 (Fig. 2, panels 3), and then photocleaved for further MS analysis yielding a single peak at m/z 6,255 (product **4**) (Fig. 2, panels 4). The third extension using dATP-PC-ROX to yield 5′—UGA(PC-ROX)-3′ (**5**), the fourth extension using dCTP-PC-Bodipy-650 to yield 5′—UGAC(PC-Bodipy-650)-3′ (**7**), and their photocleavage to yield products **6** and **8** were similarly carried out and analyzed by MALDI-TOF MS as shown in Figs. 2 (5), 2 (6), 2 (7), and 2 (8). These results demonstrate that the above-synthesized four photocleavable fluorescent nucleotide analogues can successfully incorporate into the growing DNA strand in a polymerase reaction, and that the fluorophore can be efficiently cleaved by near-UV irradiation, making it feasible to use them for SBS on a chip.

The photocleavable fluorescent nucleotide analogues were then used in an SBS reaction to identify the sequence of the DNA template immobilized on a solid surface as shown in Fig. 3. A site-specific 1,3-dipolar cycloaddition coupling chemistry was used to covalently immobilize the azido-labeled double-stranded PCR products on the alkynyl-functionalized surface in the presence of a Cu(I) catalyst. Previously, we have shown that DNA is successfully immobilized on the glass surface by this chemistry and have evaluated the functionality of the surface-bound DNA and the stability of the array using a primer extension reaction (16). The surface-immobilized double-stranded PCR product was denatured by using a 0.1 M NaOH solution to remove the complementary strand without the azido group, thereby generating a single-stranded PCR template on the surface. Then, a 5′-phosphorylated self-priming moiety (loop primer) was ligated to the 3′ end of the above single-stranded DNA template by using *Taq*DNA ligase (21). The structure of the loop primer was designed to bear a thermally stable loop (22) and stem sequence with a melting temperature of 89°C. The 12-bp overhanging portion of the loop primer was made complementary to the 12-bp sequence of the template at its 3′ end to allow the *Taq*DNA ligase to seal the nick between the

5′-phosphate group of the loop primer and the 3′-hydroxyl group of the single-stranded DNA template. This ligation reaction produces a unique DNA moiety that can self-prime for the synthesis of a complementary strand. The ligation was found to be in quantitative yield in a parallel solution-phase reaction using the same primer and single-stranded DNA template.

The principal advantage offered by the use of a self-priming moiety as compared with the use of separate primers and templates is that the covalent linkage of the primer to the template in the self-priming moiety prevents any possible dissociation of the primer from the template under vigorous washing conditions. Furthermore, the possibility of mispriming is considerably reduced, and a universal loop primer can be used for all of the templates, allowing enhanced accuracy and ease of operation. We performed SBS on the chip-immobilized DNA template using the four photocleavable fluorescent nucleotide analogues, and the results are shown in Fig. 4. The structure of the self-priming DNA moiety is shown schematically in Fig. 4 *Upper*, with the first 12-nt sequence immediately after the priming site. The sequencing reaction on the chip was initiated by extending the self-priming DNA with dATP-PC-ROX (complementary to the T on the template) and Thermo Sequenase DNA polymerase. After washing, the extension of the primer by a single fluorescent nucleotide was confirmed by observing an orange signal (the emission signal from ROX) in a microarray scanner (Fig. 4, panels 1). After detection of the fluorescent signal, the surface was irradiated at 355 nm for 1 min by using an Nd-YAG laser to cleave the fluorophore. The surface was then washed, and a negligible residual fluorescent signal was detected to confirm complete photocleavage of the fluorophore (Fig. 4, panels 2). This was followed by incorporation of the next fluorescent nucleotide complementary to the subsequent base on the template. The entire process of incorporation, detection, and photocleavage was performed multiple times by using the four photocleavable fluorescent nucleotide analogues to identify 12 successive bases in the DNA template. The integrated fluorescence intensity on the spot, obtained from the scanner software, indicated that the incorporation efficiency was >90%, and >97% of the original fluorescence signal was removed by photocleavage. A negative control experiment consisting of incubating the self-priming DNA moiety with dATP-PC-ROX in the absence of DNA polymerase and washing the surface showed that negligible fluorescence remained as compared with that of Fig. 4, panels 1.

In summary, we have synthesized and characterized four photocleavable fluorescent nucleotide analogues and used them to produce four-color DNA sequencing data on a chip. These nucleotides have been shown to be excellent substrates for the DNA polymerase, and the fluorophore could be cleaved efficiently by using near-UV irradiation. This rapid photocleavage is important with respect to enhancing the speed of each cycle in SBS for high-throughput DNA analysis. We also demonstrated that a PCR-amplified DNA template can be ligated with a self-priming moiety and that its sequence can be accurately identified in a DNA polymerase reaction on a chip, indicating that a PCR product from any organism can be potentially used as a template for the SBS system in the future. The modification of the 3′-OH of the photocleavable fluorescent nucleotide with a small chemical group to allow reversible termination is reported in the companion article (17). The library of photocleavable fluorescent nucleotides reported here should also facilitate the development of single-molecule DNA sequencing approaches. Thus, by further improving the read-length and incorporation efficiency, this approach potentially can be developed into a high-throughput DNA-analysis system for biological research and medical applications.

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