Four-color DNA sequencing with 3′-*O*-modified nucleotide reversible terminators and chemically cleavable fluorescent dideoxynucleotides

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DNA sequencing by synthesis (SBS) on a solid surface during polymerase reaction can decipher many sequences in parallel. We report here a DNA sequencing method that is a hybrid between the Sanger dideoxynucleotide terminating reaction and SBS. In this approach, four nucleotides, modified as reversible terminators by capping the 3′-OH with a small reversible moiety so that they are still recognized by DNA polymerase as substrates, are combined with four cleavable fluorescent dideoxynucleotides to perform SBS. The ratio of the two sets of nucleotides is adjusted as the extension cycles proceed. Sequences are determined by the unique fluorescence emission of each fluorophore on the DNA products terminated by ddNTPs. On removing the 3′-OH capping group from the DNA products generated by incorporating the 3′-*O*-modified dNTPs and the fluorophore from the DNA products terminated with the ddNTPs, the polymerase reaction reinitiates to continue the sequence determination. By using an azidomethyl group as a chemically reversible capping moiety in the 3′-*O*-modified dNTPs, and an azido-based cleavable linker to attach the fluorophores to the ddNTPs, we synthesized four 3′-*O*-azidomethyl-dNTPs and four ddNTP-azidolinker-fluorophores for the hybrid SBS. After sequence determination by fluorescence imaging, the 3′-*O*-azidomethyl group and the fluorophore attached to the DNA extension product via the azidolinker are efficiently removed by using Tris(2-carboxyethyl)phosphine in aqueous solution that is compatible with DNA. Various DNA templates, including those with homopolymer regions, were accurately sequenced with a read length of >30 bases by using this hybrid SBS method on a chip and a four-color fluorescence scanner.

sequencing by synthesis | DNA chip

The completion of the Human Genome Project (1) was a monumental achievement in biological science. The engine behind this project was the Sanger sequencing method (2), which is still the gold standard in genome research. The prolonged success of the Sanger sequencing method is because of its efficiency and fidelity in producing dideoxy-terminated DNA products that can be separated electrophoretically and detected by fluorescence (3–5). However, a challenge in the use of electrophoresis for DNA separation is the difficulty in achieving high throughput and the complexity involved in the automation, although some level of increased parallelization may be achieved by using miniaturization (6).

To overcome the limitations of the Sanger sequencing technology, a variety of new methods have been investigated. Such approaches include sequencing by hybridization (7), mass spectrometry sequencing (8, 9), sequencing by nanopores (10), and sequencing by ligation (11). More recently, DNA sequencing by synthesis (SBS) approaches such as pyrosequencing (12), sequencing of single DNA molecules (13, 14), and polymerase colonies (15) have been widely explored. Previously, we reported the development of a general strategy to rationally design cleavable fluorescent nucleotide reversible terminators (NRTs) for four-color DNA sequencing by synthesis (16–20). In this approach, four nucleotides (A, C, G and T) are modified as NRTs by attaching a cleavable fluorophore to a specific location on the base and capping the 3′-OH with a small chemically reversible moiety so that they are still recognized as substrates by DNA polymerase. DNA templates consisting of homopolymer regions were accurately sequenced by this approach. A recently developed SBS system based on a similar design of the cleavable fluorescent NRTs has already found wide application in genome biology (21–23). In addition, we have used 3′-*O*-modified NRTs to solve the homopolymer sequencing problem in conventional pyrosequencing (24).

We report here an alternative sequencing method that is a hybrid between the Sanger dideoxy chain-terminating reaction and SBS, and discuss the advantages that come with this hybrid approach. The fundamental difference between the two methods is that the Sanger method produces every possible complementary DNA extension fragment for a given DNA template and obtains the sequence after the separation and detection of these fragments, whereas SBS relies on identification of each base as the DNA strand is extended by cleavable fluorescent NRTs that temporarily pause the DNA synthesis for sequence determination. The limiting factor for increasing throughput in the Sanger method is the requirement to use electrophoresis.

Challenges in using SBS with cleavable fluorescent NRTs involve the further improvement of the DNA polymerase that efficiently recognizes the modified nucleotides. In addition, the first generation of the cleavable fluorescent NRTs synthesize the DNA strand with a propargyl amino group modification on the base during SBS (20), which might interfere with the activity of the polymerase for chain elongation. The advantage of the Sanger method is the dideoxy chain fragment-producing reaction. Once the DNA strand is terminated by incorporation of a fluorescent dideoxynucleotide, it is no longer involved in further DNA extension reactions. Therefore, the continuous DNA polymerase extension reaction occurs with only natural nucleotides at high efficiency leading to a read length of >700 bp. The most attractive feature in the SBS approach is the massive

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parallel readout capability by using a high-density DNA chip without the need to separate the DNA products. We have explored the integration of the advantageous features of the two methods to develop a hybrid DNA sequencing approach. In this method, four nucleotides, modified as reversible terminators by capping the 3′-OH with a small reversible moiety so that they are still recognized as substrates by DNA polymerase, are combined with four cleavable fluorescent dideoxynucleotides to perform SBS. DNA sequences are determined by the unique fluorescence emission of each fluorophore on the DNA products terminated by ddNTPs. On removing the 3′-OH capping group from the DNA products generated by incorporating the 3′-*O*-modified dNTPs and the fluorophore from the DNA products terminated with the ddNTPs, the polymerase reaction reinitiates to continue the sequence determination [supporting information (SI) Fig. S1].

By using an azidomethyl group as a chemically reversible capping moiety in the 3′-*O*-modified dNTPs, and an azido-based cleavable linker to attach the fluorophores to ddNTPs, we synthesized four 3′-*O*-azidomethyl-dNTPs (3′-*O*-N_3-dNTPs) and four ddNTP-azidolinker-fluorophores (ddNTP-N_3-fluorophores) for the hybrid SBS. The azidomethyl capping moiety on the 3′-OH group and the cleavable fluorophore on the DNA extension products are efficiently removed after fluorescence detection for sequence determination by using a chemical method that is compatible with DNA. Various DNA templates, including those with homopolymer regions, were accurately sequenced with a read length of >30 bases by using this hybrid SBS method.

Results and Discussion

Design and Synthesis of 3′-*O*-Modified NRTs and Cleavable Fluorescent Dideoxynucleotide Terminators for the Hybrid SBS. A critical requirement for using SBS methods to unambiguously sequence DNA is a suitable chemical moiety to cap the 3′-OH of the nucleotide such that it temporarily terminates the polymerase reaction to allow the identification of the incorporated nucleotide. A stepwise addition of separate nucleotides with a free 3′-OH group has inherent difficulties in detecting sequences in homopolymeric regions (12, 13). Capping the 3′-OH group of the nucleotides with a reversible moiety allows for the addition of all four nucleotides simultaneously in performing SBS, thereby increasing accuracy and reducing the number of cycles needed. However, it is essential that the capping group be efficiently removed from the DNA extension products to regenerate the 3′-OH group for continuous polymerase reactions. Our previous research efforts have firmly established the molecular level strategy to rationally modify the nucleotides by capping the 3′-OH with a small chemically reversible moiety for SBS (16–20). Building on our successful 3′-*O*-modification strategy for the synthesis of the NRTs, we have explored alternative chemically reversible groups for capping the 3′-OH of the nucleotides. In 1991, Zavgorodny *et al.* (25) reported the capping of the 3′-OH group of the nucleoside with an azidomethyl moiety, which can be chemically cleaved under mild condition with triphenylphosphine. Various 3′-*O*-azidomethyl nucleoside analogues have subsequently been synthesized (26). Cleavable fluorescent NRTs with a variety of 3′-*O*-modification groups, including the azidomethyl moiety, have been recently proposed for SBS (27), following the general principle that we reported (16, 18, 20) to design the NRTs that can be recognized as substrates for DNA polymerase by attaching a cleavable fluorophore to a specific location on the base and capping the 3′-OH with a small chemically reversible moiety.

We synthesized and evaluated four 3′-*O*-azidomethyl-modified NRTs (3′-*O*-N_3-dNTPs) (Fig. 1) for the hybrid SBS. The 3′-*O*-modified NRTs containing an azidomethyl group to cap the 3′-OH on the ribose ring were synthesized based on a

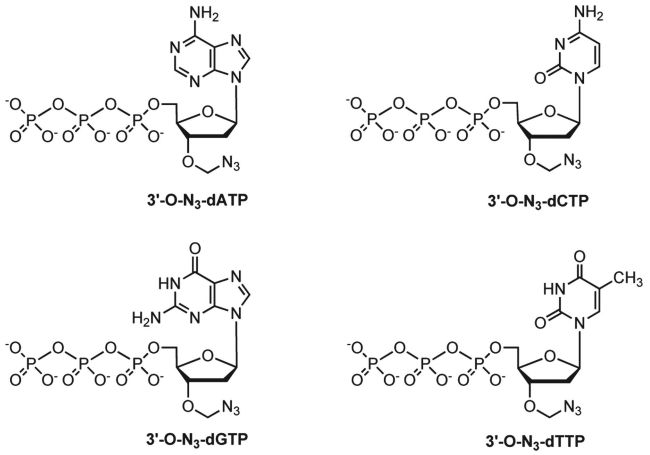

Fig. 1. Structures of the nucleotide reversible terminators, 3′-*O*-N_3-dATP, 3′-*O*-N_3-dCTP, 3′-*O*-N_3-dGTP, and 3′-*O*-N_3-dTTP.

method similar to that reported by Zavgorodny *et al.* (25, 26) as described in *SI Text*. The 3′-*O*-azidomethyl group on the DNA extension product generated by incorporating each of the NRTs is efficiently removed by the Staudinger reaction by using aqueous Tris(2-carboxyethyl) phosphine (TCEP) solution (28, 29) followed by hydrolysis to yield a free 3′-OH group for elongating the DNA chain in subsequent cycles of the hybrid SBS (Fig. S2*A*).

To demonstrate the feasibility of carrying out the hybrid SBS on a DNA chip, we designed and synthesized 4 cleavable fluorescent dideoxynucleotides, ddNTP-N_3-fluorophores (ddCTP-N_3-Bodipy-FL-510, ddUTP-N_3-R6G, ddATP-N_3-ROX, and ddGTP-N_3-Cy5) (Fig. 2). The ddNTP-N_3-fluorophores will be combined with the 4 NRTs (Fig. 1) to perform the hybrid SBS. 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) (30). Thus, we attached each unique fluorophore to the 5 position of C/U and the 7 position of A/G through a cleavable linker, which is also based on an azido-modified moiety (29) as a trigger for cleavage, a mechanism that is similar to the removal of the 3′-*O*-azidomethyl group (Fig. S2*B*). The synthesis and characterization of the cleavable fluorescent dideoxynucleotides in Fig. 2 are described in *SI Text*. The ddNTP-N_3-fluorophores are found to efficiently incorporate into the growing DNA strand to terminate DNA synthesis for sequence determination. The fluorophore on a DNA extension product, which is generated by incorporation of the cleavable fluorescent ddNTPs, is removed rapidly and quantitatively by TCEP from the DNA extension product in aqueous solution.

Continuous Polymerase Extension by Using 3′-*O*-Modified NRTs and Characterization by MALDI-TOF Mass Spectrometry. To verify that the 3′-*O*-N_3-dNTPs incorporate accurately in a base-specific manner in the polymerase reaction, four continuous DNA extension and cleavage reactions were carried out in solution by using 3′-*O*-N_3-dNTPs as substrates. This allowed the isolation of the DNA product at each step for detailed molecular structure characterization as shown in Fig. 3. The first extension product 5′-primer-C-N_3-3′ (**1**) was desalted and analyzed by using MALDI-TOF MS (Fig. 3*A*). This product was then incubated in aqueous TCEP solution to remove the azidomethyl moiety to yield the cleavage product (**2**) with a free 3′-OH group, which was also analyzed by using MALDI-TOF MS (Fig. 3*B*). As can be seen from Fig. 3*A*, the MALDI-TOF MS spectrum consists of a distinct peak corresponding to the DNA extension product

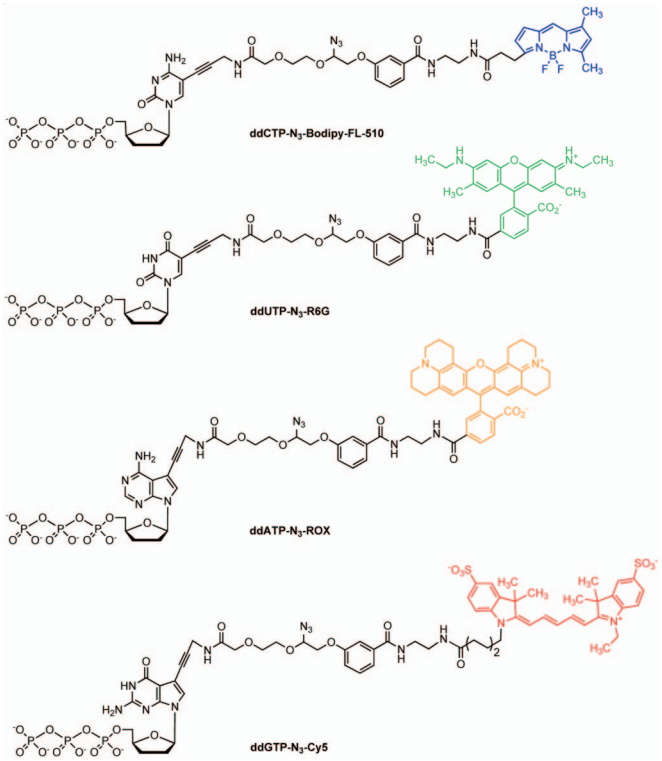

Fig. 2. Structures of the cleavable fluorescent ddNTPs: ddCTP-N_3-Bodipy-FL-510 ($\lambda_{abs\ (max)}$ = 502 nm; $\lambda_{em\ (max)}$ = 510 nm), ddUTP-N_3-R6G ($\lambda_{abs\ (max)}$ = 525 nm; $\lambda_{em\ (max)}$ = 550 nm), ddATP-N_3-ROX ($\lambda_{abs\ (max)}$ = 585 nm; $\lambda_{em\ (max)}$ = 602 nm), and ddGTP-N_3-Cy5 ($\lambda_{abs\ (max)}$ = 649 nm; $\lambda_{em\ (max)}$ = 670 nm).

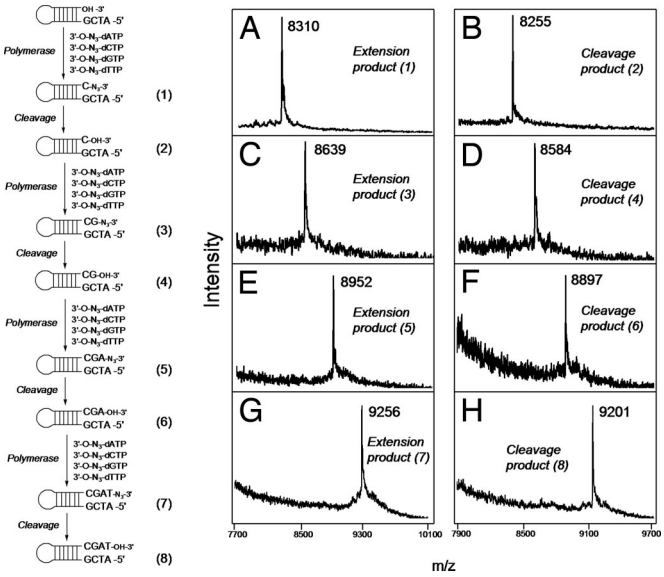

Fig. 3. The polymerase extension scheme (*Left*) and MALDI-TOF MS spectra of the four consecutive extension products and their cleavage products (*Right*) using four 3′-*O*-N_3-dNTPs. Primer extended with 3′-*O*-N_3-dCTP to yield product 1 (*A*), and its cleavage product 2 (*B*); product 2 extended with 3′-*O*-N_3-dGTP to yield product 3 (*C*), and its cleavage product 4 (*D*); product 4 extended with 3′-*O*-N_3-dATP to yield product 5 (*E*), and its cleavage product 6 (*F*); product 6 extended with 3′-*O*-N_3-dTTP to yield product 7 (*G*), and its cleavage product 8 (*H*). The azidomethyl moiety capping the 3′-OH of the DNA extension products is completely removed by TCEP aqueous solution to continue the polymerase reaction.

5′-primer-C-N_3-3′ (**1**) (*m/z* 8,310), which confirms that the NRT is incorporated base-specifically by DNA polymerase into a growing DNA strand. Fig. 3*B* shows the cleavage result on the DNA extension product. The extended DNA mass peak at *m/z* 8,310 completely disappeared, whereas the peak corresponding to the cleavage product 5′-primer-C-3′ (**2**) appears as the sole dominant peak at *m/z* 8,255, which establishes that TCEP incubation completely cleaves the 3′-*O*-azidomethyl group with high efficiency. The next extension reaction was carried out by using this cleaved product, which now has a free 3′-OH group, as a primer to yield a second extension product, 5′-primer-CG-N_3-3′ (**3**) (*m/z* 8,639; Fig. 3*C*). As described above, the extension product (**3**) was cleaved to generate product (**4**) for further MS analysis yielding a single peak at *m/z* 8,584 (Fig. 3*D*). The third extension reaction to yield 5′-primer-CGA-N_3-3′ (**5**) (*m/z* 8,952; Fig. 3*E*), the fourth extension to yield 5′-primer-CGAT-N_3-3′ (**7**) (*m/z* 9,256; Fig. 3*G*) and their cleavage to yield products (**6**) (*m/z* 8,897; Fig. 3*F*) and (**8**) (*m/z* 9,201; Fig. 3*H*) were similarly carried out and analyzed by MALDI-TOF MS. These results demonstrate that all four 3′-*O*-N_3-dNTPs are successfully synthesized and efficiently incorporated base-specifically into the growing DNA strand in a continuous polymerase reaction as reversible terminators and the 3′-OH capping group on the DNA extension products is quantitatively cleaved by TCEP.

Polymerase Extension by Using Cleavable Fluorescent Dideoxynucleotide Terminators and Characterization by MALDI-TOF Mass Spectrometry. To verify that the four cleavable fluorescent ddNTPs (ddCTP-N_3-Bodipy-FL-510, ddUTP-N_3-R6G, ddATP-N_3-ROX, and ddGTP-N_3-Cy5) (Fig. 2) are incorporated accurately in a base-specific manner in a polymerase reaction, single-base extension reactions with four different self-priming DNA templates whose next complementary base was either A, C, G, or T were carried out in solution. After the reaction, the four different primer extension products were analyzed by MALDI-TOF MS as shown in Fig. 4. Single clear mass peaks at 9,180, 8,915, 9,317, and 9,082 (*m/z*) corresponding to each primer extension product with no leftover starting materials were produced by using ddNTP-N_3-fluorophores (Fig. 4 *A*, *C*, *E*, and *G*). Brief incubation of the DNA extension products in an aqueous TCEP solution led to the cleavage of the linker tethering the fluorophore to the dideoxynucleotide. Fig. 4 *B*, *D*, *F*, and *H* shows the cleavage results for the DNA products extended with ddNTP-N_3-fluorophores. The mass peaks at 9,180, 8,915, 9,317, and 9,082 (*m/z*) have completely disappeared, whereas single peaks corresponding to the cleavage products appear at 8,417, 8,394, 8,433, and 8,395 (*m/z*), respectively. These results demonstrate that cleavable fluorescent ddNTPs are successfully synthesized and efficiently terminated the DNA synthesis in a polymerase reaction and that the fluorophores are quantitatively cleaved by TCEP. Thus, these ddNTP analogues meet the key requirements necessary for performing the hybrid SBS in combination with the NRTs.

Four-Color DNA Sequencing on a Chip by Using Cleavable Fluorescent Dideoxynucleotides and 3′-*O*-Modified NRTs by the Hybrid SBS Approach. In our four-color hybrid SBS approach, the identity of the incorporated nucleotide is determined by the unique fluorescence emission from the four fluorescent dideoxynucleotides, whereas the role of the 3′-*O*-modified NRTs is to further extend the DNA strand. Therefore, the ratio of the ddNTP-N_3-fluorophores and 3′-*O*-N_3-dNTPs during the polymerase reaction determines how much of the ddNTP-N_3-fluorophores incorporate and, thus, the corresponding fluorescence emission strength. With a finite amount of immobilized DNA template on a solid surface, initially the majority of the priming strands should be extended with 3′-*O*-N_3-dNTPs, whereas a relatively smaller amount should be extended with ddNTP-N_3-fluoro-

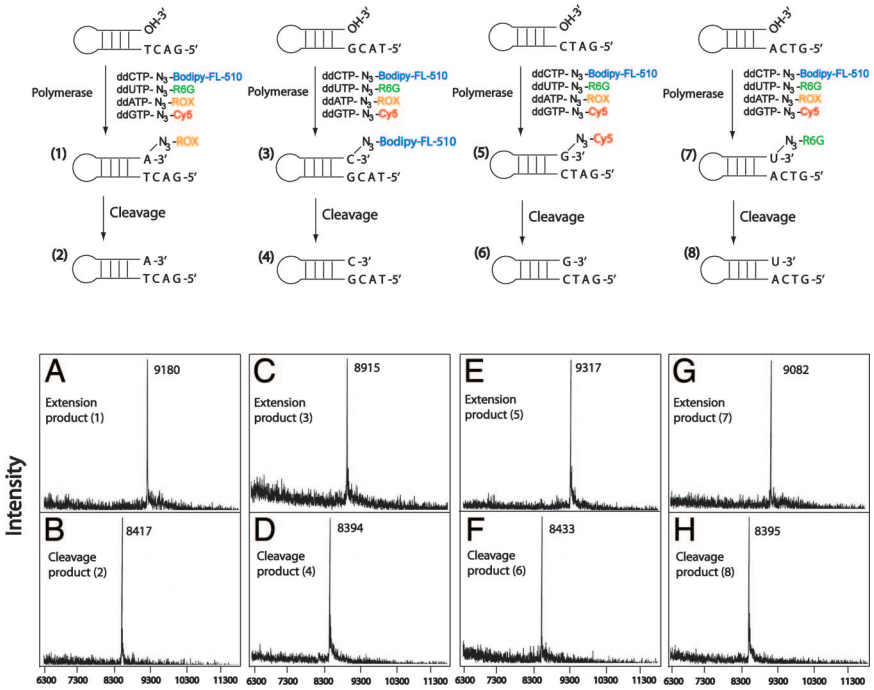

Fig. 4. A polymerase reaction scheme *(Top)* to yield DNA extension products by incorporating each of the four ddNTP-N_3-fluorophores and the subsequent cleavage reaction to remove the fluorophores from the DNA extension products. MALDI-TOF MS spectra (*Bottom*) showing efficient base-specific incorporation of the ddNTP-N_3-fluorophores and the subsequent cleavage of the fluorophores from the DNA extension products: (*A*) Primer extended with ddATP-N_3-ROX (1) (peak at 9,180 *m/z*), (*B*) its cleavage product 2 (8,417 *m/z*); (*C*) primer extended with ddCTP-N_3-Bodipy-FL-510 (3) (peak at 8,915 *m/z*), (*D*) its cleavage product 4 (8,394 *m/z*); (*E*) primer extended with ddGTP-N_3-Cy5 (5) (peak at 9,317 *m/z*), (*F*) its cleavage product 6 (8,433 *m/z*); (*G*) primer extended with ddUTP-N_3-R6G (7) (peak at 9,082 *m/z*), and (*H*) its cleavage product 8 (8,395 *m/z*).

phores to produce sufficient fluorescent signals that are above the fluorescence detection system's sensitivity threshold for sequence determination. As the sequencing cycle continues, the amount of the ddNTP-N_3-fluorophores needs to be gradually increased to maintain the fluorescence emission strength for detection. Following these guidelines, we performed the hybrid SBS on a chip-immobilized DNA template by using the 3′-*O*-N_3-dNTP/ddNTP-N_3-fluorophore combination and the results are shown in Fig. 5. The general four-color sequencing reaction scheme on a DNA chip is shown in Fig. 5*A*.

The *de novo* sequencing reaction on the chip was initiated by extending the self-priming DNA by using a solution consisting of four 3′-*O*-N_3-dNTPs and four ddNTP-N_3-fluorophores, and 9°N DNA polymerase. The hybrid SBS allows for the addition of all eight nucleotide substrates simultaneously to unambiguously determine DNA sequences. This reduces the number of steps needed to complete the sequencing cycle, while increasing the sequencing accuracy because of competition among the substrates in the polymerase reaction. The DNA products extended by ddNTP-N_3-fluorophores, after fluorescence detection for sequence determination and cleavage, are no longer involved in the subsequent polymerase reaction cycles because they are permanently terminated. Therefore, further polymerase reaction only occurs on a DNA strand that incorporates the 3′-*O*-N_3-dNTPs, which subsequently turn back into natural nucleotide on cleavage of the 3′-OH capping group, and should have no deleterious effect on the polymerase binding to incorporate subsequent nucleotides for growing the DNA chains. However, successive addition of the previously designed cleavable fluorescent NRTs (20, 27, 29) into a growing DNA strand during SBS leads to a newly synthesized DNA chain with a leftover propargyl amino group at each nucleobase. This may interfere with the ability of the enzyme to efficiently incorporate the next incoming nucleotide, which will lead to loss of synchrony and thereby reduction in the read length. This challenge might potentially be overcome by reengineering DNA polymerases that efficiently recognize and accept the modified DNA strand, or by alternative design of the fluorescent NRTs (31).

To negate any lagging fluorescence signal that is caused by a previously unextended priming strand, a synchronization step was added to reduce the amount of unextended priming strands after the initial extension reaction shown in the scheme of Fig. 5*A*. A synchronization reaction mixture consisting of just the four 3′-*O*-N_3-dNTPs in relatively high concentration was used along with the 9°N DNA polymerase to extend any remaining priming strands that retain a free 3′-OH group to synchronize the incorporation.

The four-color images from a fluorescence scanner for each step of the hybrid SBS on a chip is shown in Fig. 5*B*. The first extension of the primer by the complementary fluorescent ddNTP, ddCTP-N_3-Bodipy-FL-510, was confirmed by observing a blue signal (the emission from Bodipy-FL-510) [Fig. 5*B* (1)]. After fluorescent signal detection, the surface was immersed in a TCEP solution to cleave both the fluorophore from the DNA product extended with ddNTP-N_3-fluorophores and the 3′-*O*-azidomethyl group from the DNA product extended with 3′-*O*-N_3-dNTPs. The surface of the chip was then washed, and a negligible residual fluorescent signal was detected, confirming cleavage of the fluorophore [Fig. 5*B* (2)]. This was followed by another extension reaction with the 3′-*O*-N_3-dNTP/ddNTP-N_3-fluorophore solution to incorporate the next nucleotide complementary to the subsequent base on the template. The entire process of incorporation, synchronization, detection, and cleavage was performed multiple times to identify 32 successive bases in the DNA template. The plot of the fluorescence intensity vs. the progress of sequencing extension (raw four-color sequencing data) is shown in Fig. 5*C*. The DNA sequences including the homopolymer regions are unambiguously identified with no

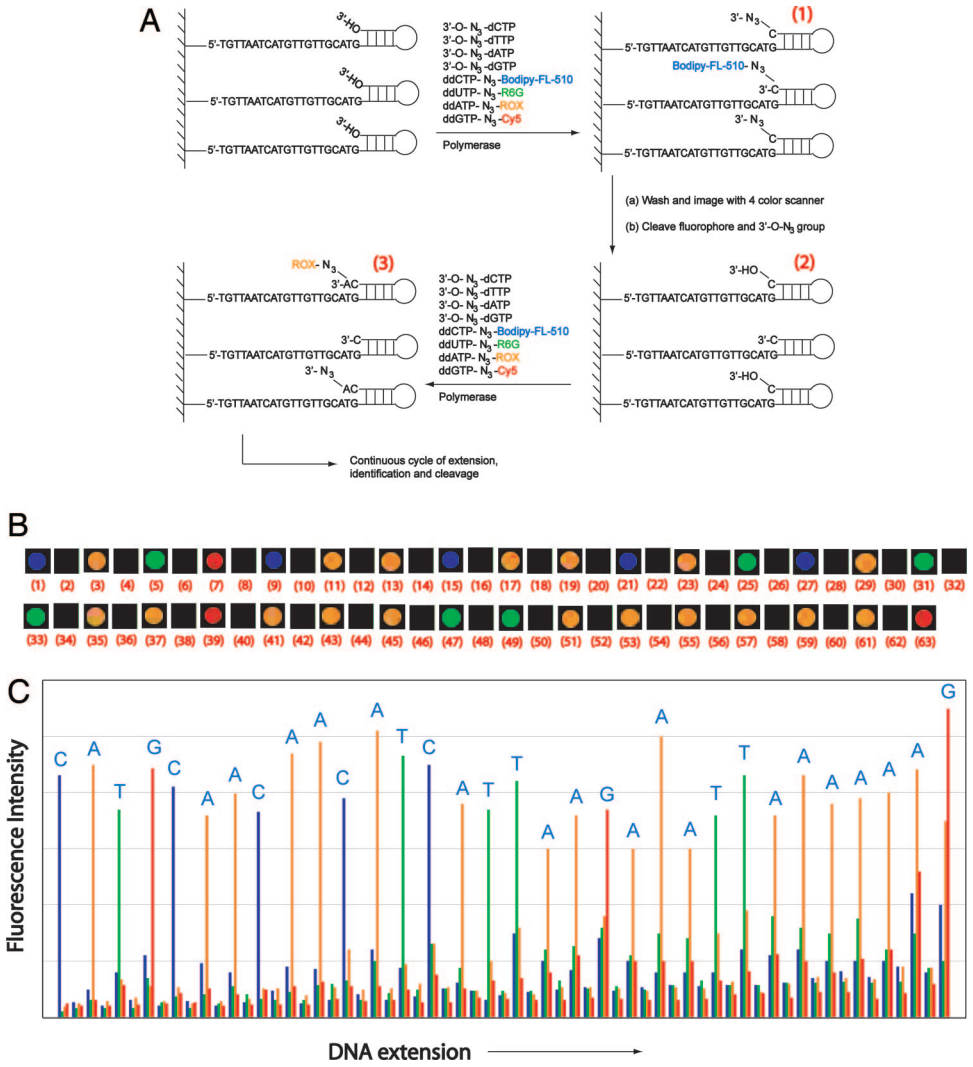

Fig. 5. Four-color DNA sequencing by the hybrid SBS. (*A*) A hybrid SBS scheme for four-color sequencing on a chip by using four 3′-*O*-N_3-dNTPs and four ddNTP-N_3-fluorophores. (*B*) The four-color fluorescence images for each step of the SBS: (1) incorporation of 3′-*O*-N_3-dCTP and ddCTP-N_3-Bodipy-FL-510; (2) cleavage of N_3-Bodipy-FL-510 and 3′-CH_2N_3 group; (3) incorporation of 3′-*O*-N_3-dATP and ddATP-N_3-Rox; (4) cleavage of N_3-ROX and 3′-CH_2N_3 group; (5) incorporation of 3′-*O*-N_3-dTTP and ddUTP-N_3-R6G; (6) cleavage of N_3-R6G and 3′-CH_2N_3 group; (7) incorporation of 3′-*O*-N_3-dGTP and ddGTP-N_3-Cy5; (8) cleavage of N_3-Cy5 and 3′-CH_2N_3 group; images 9–63 are similarly produced. (*C*) A plot (four-color sequencing data) of raw fluorescence emission intensity obtained by using 3′-*O*-N_3-dNTPs and ddNTP-N_3-fluorophores. The small groups of peaks between the identified bases are fluorescent background from the DNA chip.

errors from the four-color raw fluorescence data without any processing. Similar four-color sequencing data were obtained for a variety of DNA templates (Fig. S3).

Conclusion

We have synthesized four 3′-*O*-N_3-dNTPs along with four cleavable fluorescent ddNTPs and used them to produce four-color *de novo* DNA sequencing data on a chip by the hybrid SBS approach that has the following advantages. With the 3′-*O*-N_3-dNTPs, after cleavage of the 3′-OH capping group of the DNA extension product, there are no traces of modification left on the growing DNA strand. Therefore, there will be no adverse effect on the DNA polymerase for the incorporation of the next complementary nucleotide. Second, the cleavable fluorescent ddNTPs and 3′-*O*-N_3-dNTPs are permanent and reversible terminators, respectively, which allow the interrogation of each base in a serial manner, a key strategy enabling accurate determination of homopolymeric regions of DNA. In addition, because all of the steps of the nucleotide incorporation, fluorescence detection for sequence determination, cleavage of the fluorophore, and the 3′-*O*-azidomethyl group are performed on a DNA chip, there is no longer a need for electrophoretic DNA fragment separation as in the classical Sanger sequencing method.

We have experimentally determined the ratio of the 3′-*O*-N_3-dNTPs and ddNTP-N_3-fluorophores to yield sequencing read length of 32 bases. The signal strength at base 32 is as strong as that of the first base (Fig. 5*C*), indicating it should be possible to increase the read length of the hybrid SBS further by optimizing the extension conditions to reduce the background fluorescence in the later sequencing cycles. The ultimate read length of this hybrid SBS system depends on three factors: the number of starting DNA molecules on each spot of a DNA chip, the reaction efficiency, and the detection sensitivity of the system. The read length with the Sanger sequencing method commonly reaches >700 bp. The hybrid SBS approach described here may have the potential to reach this read length, especially with improvements in the sensitivity of the fluorescent detection system, where single molecules can be reliably detected.

With sequencing read length from 14 to 30 bases in the next generation DNA sequencing systems, massive parallel digital

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