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Propargylamine-selective dual fluorescence turn-on method for post-synthetic labeling of DNA

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We have developed a propargylamine-selective dual fluorescence turn-on system, using ylidenmalononitrile enamines, for post-synthetic DNA labeling, allowing the direct monitoring of DNA using dual emission in living Cell.

Fluorescence DNA labeling has been applied in many bioimaging studies,[1–3] including investigations of the dynamics of intracellular DNA[4-5] and RNA,[6–8] DNA structural transformations,[9–13] gene expression,[14-16] and various gene diagnostics.[17–18] Although many methods have been developed, post-synthetic fluorescence labeling would be the most efficient biocompatible method. Over the several years numerous post synthetic labeling method have been reported using inverse electron demand Diels–Alder (iEDDA) reactions,[19-20] diazyrin based photo cross linking,[21-22] and Suzuki–Miyaura cross coupling for RNA labeling.[23]

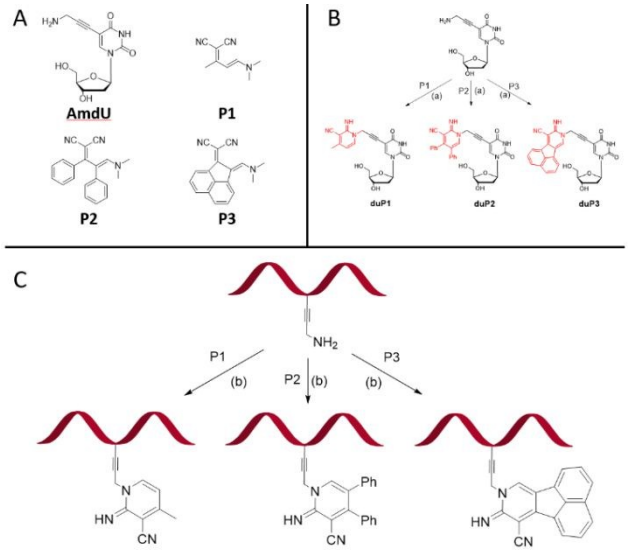
Ideally, post-synthetic fluorescence DNA labeling would result in fluorescence properties appearing only when the target DNA is labeled, and not on prior to its labeling. Furthermore, ideal post-synthetic fluorescence DNA labeling should be shown dual emission that provide accurate localization and eliminate the interrupted background signal in *in vivo* imaging.

Most commercially available fluorescence labeling methods use compounds that already exhibit strong single fluorescence prior to labeling of the DNA[32-38] such that some fluorescent residues can remain after labeling to elicit false-positive signals. In this point of view, post synthetic fluorescence turn on system would be promising labeling method.

A lot of fluorescence turn on system on DNA have been developed using tetrazine-modified dyes[24-27] and azide[28] and alkyne-modified dyes based on coumarins,[29-30] and naphthalimides.[31] However it still demands more diverse types of fluorescence turn on system with different functinal group. Propargyl amine directing fluorescence turn on system would be alternative labeling method instead of tetrazine and azide based fluorescence turn on system.

In this regard, our goal for this study was to develop a bioorthogonal reaction mediated dual fluorescence turn-on method using an amino-functionalized DNA. We wanted our compounds to display no (or very weak) fluorescence prior to labeling, yet strong dual fluorescence afterward. This bioorthogonal reaction mediated fluorescence turn-on method would, thereby, minimize false-positive signals while simplifying the labeling process and providing a convenient means of monitoring the fluorescence signal directly.

For this purpose, we employed the reaction between ylidenemalononitrile enamines and primary amines, as developed previously by the McQuade group.[39,40] Our strategy for developing nucleoside- and oligonucleotide-based fluorescence turn-on systems was to use ylidenemalononitrile enamines and amino-presenting nucleosides or oligonucleotides (**Scheme 1**). The functionalization of oligonucleotides can be difficult because these complex structured macrobiomolecules often hide their reaction sites. Thus, we suspected that a propargylamino group would be sufficiently long to ensure the post-reaction labeling of the oligonucleotides with ylidenemalononitrile enamines.



Scheme 1. (A) Structures of 5′-(propargylamino)deoxyuridine (AmdU) and the ylidenemalononitrile enamines tested in this study. (B) Design and synthesis of fluorescence nucleosides. (C) Synthesis of functionalized oligonucleotides.

As a potentially suitable amino-derived nucleoside, we synthesized 5′-(propargylamino) deoxyuridine (AmdU). Next, we reacted AmdU

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with three different ylidenemalononitrile enamines (**P1**–**P3**) and obtained the three novel deoxyuridine-based nucleosides **duP1**, **duP2**, and **duP3**, the structures of which we confirmed using NMR spectroscopy and high-resolution mass spectrometry (**Schemes S1** - **S5**).

When we examined the photophysical properties of the three new nucleoside derivatives (**duP1**–**duP3**), we found, gratifyingly, that they all displayed high fluorescence despite their precursors **P1**–**P3** not exhibiting fluorescence or only weak fluorescence (**Figure 1**). Each of the ylidenemalononitrile enamine–reacted nucleosides **duP1**–**duP3** exhibited different fluorescence properties: **duP1** displayed fluorescence at λ_{max}=470 nm (blue-green); **duP2** at λ_{max} = 525 nm (green); and **duP3**, interestingly, exhibited dual emission at λ_{max}= 476 nm (λ_{ex} = 420nm) and at λ_{max}= 570 nm (λ_{ex} = 510 nm). Measurements of the fluorescence quantum yields of these three new fluorescent nucleoside derivatives revealed that **duP3** had a much higher quantum yield (Φ = 0.30) than those of **duP2** (Φ = 0.02) and **duP1** (Φ = 0.001) (**Table S2**).

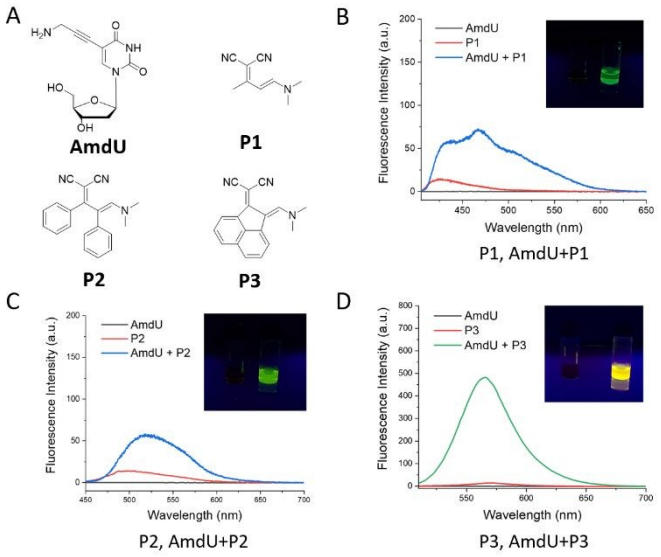

Figure 1. Fluorescence spectra and photographs (under irradiation at 365 nm) of the products of the reactions of AmdU with the ylidenemalononitrile enamines (B) **P1**, (C) **P2**, and (D) **P3** in MeCN, stirred overnight at room temperature.

The fluorescence properties of **duP1**–**duP3** varied depending on the solvent. Although **duP1** did not undergo any significant change in fluorescence upon changing the solvent from MeCN to water (**Figure S1**), the fluorescence of **duP2** did change accordingly from blue to green (**Figure S2**), while **duP3** underwent a dramatic change in color from blue in water to red in various other solvents (**Figure S3**). Based on its high quantum yield with dual emission which is useful to verify the location of fluorescence in the living cell and high sensitivity toward the solvent, we suspected that **duP3** would be promising fluorescent nucleoside for incorporation into DNA.

Next, we investigated whether this fluorescence turn-on process operated also with oligonucleotides, with the possibility that the amino groups of deoxycytosine, deoxyadenosine, and deoxyguanosine residues might compete with the propargylamino group. With this concern in mind, we synthesized a trifluoroacetyl-protected propargylamino-deoxyuridine phosphoramidite (**Scheme S1**) and incorporated it into various oligonucleotides using solid phase synthesis, then deprotected the propargylamino group (using ammonium hydroxide) and applied general oligonucleotide synthetic and purification procedures to obtain free propargylamino-presenting oligonucleotide sequence (**ODN 1**) (**Table S1**). We confirmed the propargylamino oligonucleotide using MALDI-TOF mass spectrometry (**Figure S7**).

We chose the **P3**, which induced promising fluorescence properties when it forms the nucleoside **duP3**, for reaction with propargylamino-presenting oligonucleotide. The optimized reaction conditions involving MeCN and phosphate buffer (1:1) as the solvent system and a ratio between the propargylamino-presenting oligonucleotide and **P3** of 1:10—provided a high yield of the oligonucleotide product (**Oligo P3**), which was purified using HPLC.

We used radio-labeling method to confirm the success of this reaction because of its high sensitivity toward even small structural changes. Indeed, **Figure 2A** reveals the different mobilities of the propargylamino-presenting oligonucleotide and the products **Oligo P1**–**P3**. We confirmed the structures of these products by measuring their MALDI-TOF mass spectra (**Figures 2B** and **S8**). These data confirmed that selective reactions had occurred between the propargylamino group and the ylidenemalononitrile enamines, and that no such reactions occurred with the other free amino groups of the oligonucleotides.

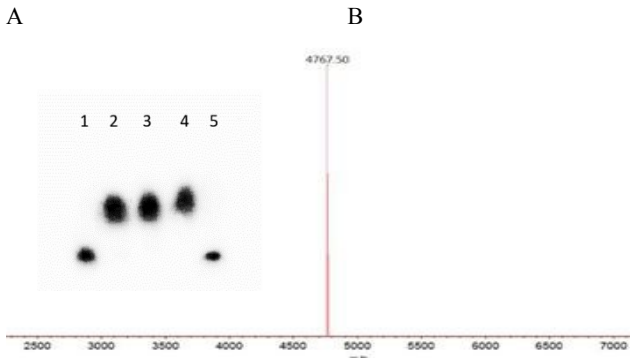

Figure 2. (A) Autoradiogram of the denaturing polyacrylamide gel electrophoresis of the reactions of ^{32}P-5′-end–labeled **ODN1** (containing 1 μM AmdU) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO_4, pH 7.4) and the three ylidenemalononitrile enamines (0.1 mM in MeCN). These mixtures had been stirred overnight. Lane 1: ^{32}P-5′-end–labeled **ODN1**; lane 2: reaction with **P1**; lane 3: reaction with **P2**; lane 4: reaction with **P3**; lane 5: ^{32}P-5′-end–labeled **ODN1**. (B) MALDI-TOF mass spectrum of the reaction product formed between **ODN1** and **P3**; **Oligo P3**: calcd *m/z* 4766.28, found 4767.50.

We recorded fluorescence spectra to examine whether the fluorescence turn-on was also occurring with the modified oligonucleotides. Interestingly, when the propargylamino-presenting oligonucleotide was added into solutions of the **P1**–**P3**, the fluorescence signals increased dramatically, with different emission wavelengths (**Figure 3A**): **Oligo P1** exhibited blue fluorescence at (λ_{max}) 475 nm; **Oligo P2** exhibited green fluorescence at (λ_{max}) 515 nm; and **Oligo P3** exhibited fluorescence at (λ_{max}) 475 nm according to a solvent effect in water. Thus, our selective fluorescence turn-on system could indeed function as a post-synthetic modification method for the direct labeling of DNA. To confirm the utility of this fluorescence turn-on system, we employed denaturing PAGE and observed the fluorescence signals directly by the naked eye at the same positions as the oligonucleotides (**Figure 3B**). The fluorescence gel image was correlated well with **Oligo P3**, with its blue fluorescence originating from the pH of the PAGE buffer. **Oligo P3** also exhibited pH-dependent fluorescence, similar to that of **duP3** (**Figure S9**).

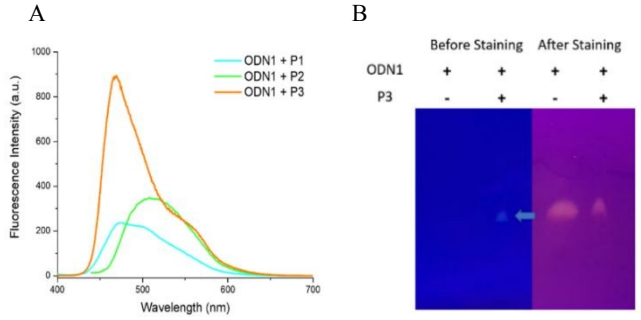

Figure 3. (A) Emission spectra of the products of the reactions of **ODN1** (0.1 mM) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with the three ylidenemalononitrile enamines (1 mM in MeCN). These reactions were stirred overnight. (B) Denaturing PAGE characterization of the product of the reaction between 0.1 mM **ODN1** (containing AmdU) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with 0.5 mM **P3** (in MeCN). Gels were stained with EtBr.

We recorded time-dependent fluorescence spectra to confirm that the turn-on system could be applied directly—using **ODN 1** (0.1 mM) and **P3**. **Figure 4** reveals a rational fluorescence increase depending on the time. This data clearly demonstrate the suitability of directly monitoring the fluorescence turn-on when applying the propargylamino-presenting oligonucleotide.

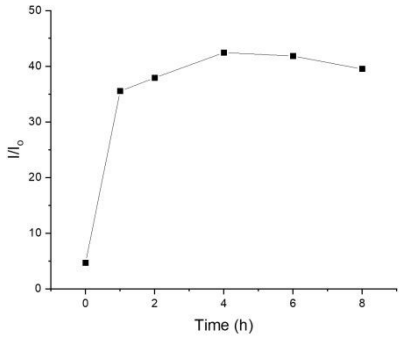

Figure 4. Time-dependent fluorescence spectra of the reaction between **ODN1** (0.1 mM) and **P3** (1 mM) in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).). I_0 means initial fluorescence and I means after fluorescence turn on at each time.

To evaluate the kinetics of the reactions, based on fluorescence emission intensity, concentration dependent studies were performed in specified interval of time (**Figure S10A-10C**). Based on results obtained value was K_m = 15.0μM/min. Upon plotting the inverse reciprocal of reactant concentrations verses time scale, we found linear relationship with k = 0. 36 mM-1min-1 (linear fitting) clearly supported that reactions followed second order kinetics (**Figure S10D**). Hence we believe that reaction followed second order kinetics under physiological conditions.

We also examined the reactivity of other amines in the nucleobases using natural oligonucleotide (**random ODN**) and **T15** that doesn't have propargyl amine (**Figure S15**). We observed **P3** reacted with only propargyl amino presenting oligonucleotide (**ODN 1**) not for the random sequence which contain several amines in their nucleobases. Most of amines in their nucleobases on the oligonucleotide must be hindered sterically and have low nucleophilicity than propargyl amine. From this result, we confirmed that the reactivity of propargyl amino presenting oligonucelotide (**ODN 1**) with **P3** is clearly discriminated from amines in the nucleobase of natural oligonucleotide.

Upon inspiring the utilities of propargylamino-presenting oligonucleotide in synthetic biology we initiated bio-orthogonal reactions in living fibroblast sarcoma-HT1080 cancer cell lines (see experimental section in supporting information).[41] Initially to identify the suitable concentration of probe **P3** per plate of live cell culture we have performed concentration dependent studies (**Figure S11**). Based on that studies residual amines present in per plate of cells cytosol were identified and background images were processed with appropriate calibration (intensity based spectral corrections) using Image J software.

Accordingly, upon incubation of 5 μM solution of **P3** in fibroblast sarcoma HT01080 cell lines, it showed very low emission in green and red channels (**Figure 5a**). In contrast upon sequential incubation of **P3** (5 μM) with small molecule propargyl amine (10.0 eq.) showed bright fluorescence emission in green and red channel and supported the amine units undergo reaction with **P3** in intracellular conditions (**Figure 5b**). Upon treatment of **P3**, with transfected propargylamino-presenting oligonucleotide using Lipofectin (TM DOTAP-DOTMA), showed bright spots in green and red channels and validated effective bioorthogonal reactions in cancer cell lines (**Figure 5c**). Higher concentration of **P3** with specified amount of propargylamino-presenting oligonucleotide showed substantial fluorescence foci spots with diffused background fluorescence (**Figure 5d**). We suspect that foci spot come from Lipofectin(TM) induced specific location of oligonucleotide in cytosol, while small molecule propargyl amine with **P3** showed random diffused fluorescence.
We confirmed the location of oligonucleotide by using blue-bright field images (**Figure S12**).

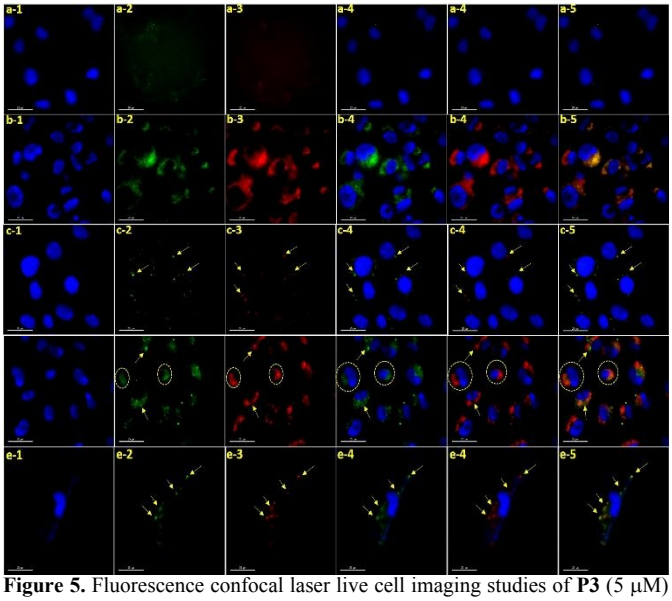

Figure 5. Fluorescence confocal laser live cell imaging studies of **P3** (5 μM) in fibroblast sarcoma-HT1080 cancer cell lines; a) Cells incubated only with **P3**, b) cells incubated with Propargyl amine (10 eq.), 60 min. followed by sequential addition of **P3** (5 μM) 60 min., c) Cells were incubated with propargylamino-presenting oligonucleotide with aid of Lipofectin (TM) (1:5 ratio), about 14 h followed by sequential addition of **P3** (3 μM) d) Cells were incubated with propargylamino-presenting oligonucleotide with aid of Lipofectin(TM) (1:5 ratio), about 14 h followed by sequential addition of increased amount of **P3** (15 μM). (validations of the background and diffused signals from the **P3** in cell cytosol); e) Cells were incubated with propargylamino-presenting oligonucleotide with aid of Lipofectin (TM) (1:5 ratio), about 14 h followed by sequential addition of **P3** (3 μM) in low cell density cell culture plate (cell counts approximately 800 to 1200 per plates). Note: Images were collected at 20 μm scale, and incubation temperature was 37° C. Each image was notified with minimum 6 consecutive readings in various locations of each plate with ~2000 cells. Images numbered from 1-6 represented blue, green, red, blue-green (merge 50:50%), blue-red (merge 50:50%) and blue-green-red channels (merge 33:33:33%) respectively.

We also examined single cell based molecular imaging upon

careful consideration of concentration effects. Propargylamino-presenting oligonucleotide were transfected in to the low cell density of cell culture plates. Accordingly, we found clear reactions between **P3** and cytosolic localized propargylamino-presenting oligonucleotide which were identified in green and red channels (**Figure 5e**). Upon careful observation through the single cell showed highly selective turn on response in green and red dual emissive channels upon incubating transfected propargylamino-presenting oligonucleotide with **P3**.

To clarify the Lipofectamine effect that also have amine, we measured fluorescence confocal laser live cell imaging using only Lipofectin(TM) with Probe **P3** based on previously reported Lipofectamine metabolism[42] and it did not induce any enough characteristic fluorescence spot (**Figure S13**). In contrast upon increment of concentration of propargylamino presenting oligoucleotide it showed high number fluorescence spots in cell cytosol, which validated the successful recognition of propargylamino-presenting oligonucleotide using **P3** (**Figure S14**).

From this result, we believe that presence of Lipofectin (TM) was almost negligible and propargylamino-presenting oligonucleotide selective fluorescence turn-on system with **P3** is operating in live fibroblast sarcoma cell line.

In conclusion, we have developed a bioorthogonal reaction mediated dual fluorescence turn-on method for post-synthetic DNA labeling using an propargylamino-functionalized DNA with ylidenmalononitrile enamines. We designed and synthesized the nucleoside derivatives **duP1**, **duP2**, and **duP3** from the reactions of propargylamino-presenting deoxyuridine and three different ylidenmalononitrile enamines, and observed dramatic fluorescence turn-on properties. Among them, **duP3** [Φ = 0.30; λ_{max} = 565 nm (reddish)]**,** interestingly, exhibited high dual emission property [λ_{ex} = 420nm and λ_{em} = 476nm (green), λ_{ex} = 510nm and λ_{em} = 570 nm (red)] with highly sensitive fluorescence depends on the solvent and pH. The site-selective reactions of a propargylamino-presenting oligonucleotide with the ylidenmalononitrile enamines also led to turn-on fluorescence in DNA, with the reactions optimized to produce the fluorescence turn- on products **Oligo P1**–**P3** (confirmed using MALDI-TOF mass spectrometry and autoradiograms with PAGE). **Oligo P3** exhibited strong fluorescence and provided a dramatic time-dependent fluorescence turn-on pattern. We also tried bioorthogonal reaction using **ODN 1** and **P3** in the living cancer cell lines and demonstrated bioorthogonal reaction mediated dual fluorescence turn-on system (green and red channel) using propargylamino-presenting oligonucleotide and **P3** in the living fibroblast sarcoma-HT1080 cancer cell lines. This system appears to be a unique method for the selective turn-on dual emission of propargylamino-presenting oligonucleotides, and should be useful for the direct and accurate monitoring of DNA in living Cell.

Acknowledgment

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Supplementary data

Electronic Supplementary Information (ESI) available online: Full experimental details; characterization of compounds; UV absorption, fluorescence and Maldi-TOF spectra of the oligonucleotides.

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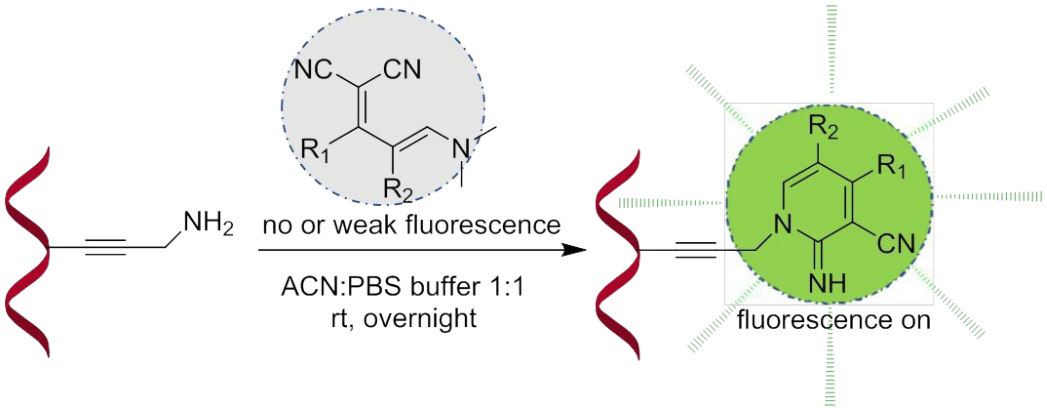

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