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A far-red emissive two-photon fluorescent probe for quantification of uracil in genomic DNA†

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We report a new method for dU detection in genomic DNA combined with UNG excision and fluorescent probe labeling. UNG can remove uracil bases to introduce abasic sites, which can react with NRNO to produce intense fluorescence because of the inhibition of the PET effect. It can also cause the polymerase extension to stop to provide details of dU site information.

Chemical modifications occur throughout genomic DNA which have significant influence on various cellular processes,[1] genome function and the conformation and stability of nucleosomes.[2] One of the most prevalent modifications is 5-methylcytosine which plays an important role in the regulation of genes,[3] genomic imprinting,[4] X chromosome inactivation, and various diseases, especially cancers.[5] Uracil, one of the nucleic acid bases present in RNA, is also frequently found in DNA resulting from the deamination of cytosine or from a misincorporation of dUMP during DNA replication.[6] In the most extreme cases of specific bacteriophages, such as in *Bacillus subtilis* PBS1 and PBS2 phages, and the *Yersinia enterocolitica* ΦR1–37 phage, the phage DNA contains deoxyuridine but no deoxythymidine.[7] Additionally, reverse-transcribed HIV genomic DNA,[8] fruit fly larvae and drosophila also showed high uracil content.[9] And uracil is recognized as an intermediate in diverse cellular processes, including intrinsic immunity against viral infection and inhibition of retrotransposition of endogenous retroelements.[10]

Genomic uracil is normally faithfully repaired by base excision repair (BER) initiated by uracil DNA glycosylases.[11] These enzymes are UNG, SMUG1, TDG and MBD4. UNG, which is encoded by the UNG gene and consists of mitochondrial UNG1 and nuclear UNG2, has a central function in the removal of dU from misincorporated dUMP residues.[12] SMUG1 has been proposed to aim at the removal of deaminated cytosine residues.[13] While TDG and MBD4 may have specialized roles in the repair of mismatched uracil, thymine and some damaged pyrimidines in double-stranded DNA.[14]

Up to now, a lot of specific approaches have been explored to locate and quantify uracil in DNA. Differential DNA denaturation PCR and ligation-mediated PCR provided uracil detection in U:G mismatches and short uracil-containing DNA fragments with excision of UNG and APE1.[15] Current genome-wide uracil mapping technology revealed that uracil varied significantly in *Escherichia coli* and budding yeast and was at a high level in human centromeric DNA, especially the binding regions of the centromere-specific histone CENP-A.[16] In addition, genomic uracil quantification methods have also been reported. LC/MS/MS based methods[17] introduced sensitive uracil quantification in *Escherichia coli* DNA and B cells.[18] Real-time polymerase chain reaction (PCR)-based techniques reflect uracil content within selected genomic segments in *E. coli* and MEF cells.[19] However, the methods for the direct fluorescence labelling of uracil in genomic DNA are still limited. Thus, chemical reagents that could be fluorescence switch-on and imaging in cell analysis of uracil in DNA are greatly needed.

To address this limitation, we developed a novel strategy capable of uracil quantification in genomic DNA through fluorescence detection combined with UNG excision and chemical probe labelling. In principle, uracil in DNA was firstly excised by UNG to generate abasic (AP) sites which can be easily labelled by chemical probes. The designed probes generally consist of two parts, the *o*-phenylenediamine group for reacting with the freshly generated X (X = AP) sites and the fluorescent group for detection. Due to the photoinduced electron transfer (PET) effect, *o*-phenylenediamine turns off the fluorescence of fluorophores such as naphthalimide,[20] BODIPY,[21] cyanine[22] and Nile Red.[23] After reaction with the X sites, the *o*-phenylenediamine transforms into benzimidazole and the PET effect is inhibited, inducing an increasing fluorescence signal (Scheme 1). This "turn on" strategy

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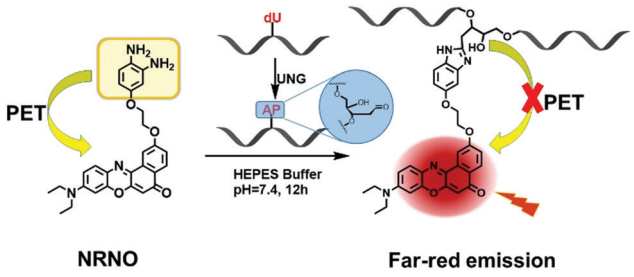

Scheme 1 A strategy for the fluorogenic labelling of dU in DNA.

due to the inhibition of the PET effect allows the selective fluorescent detection of dU sites with low background noise.

Following this design, we synthesized two probes: one is a far-red emissive two-photon (TP) excitable NRNO probe[23] and the other is a coumarin-based Cou probe (Schemes S1 and S2, ESI†). Upon the reactions with UNG treated dU containing DNA, both of these probes showed fluorescent enhancement, which indicated the feasibility of this strategy. Because of the properties of far-red emission and relatively lower background, the NRNO probe was chosen for further study (Fig. S1, ESI†).

To verify this approach in more detail, a 15-mer oligonucleotide ODN1-dU containing one dU site was used as the model to be treated by UNG for 2 h to generate ODN1-X. Both ODN1-dU and ODN1-X were incubated with NRNO under optimized conditions (50 mM HEPES buffer, pH 7.4, 60 °C, and 12 h). The RP-HPLC (monitored at 260 nm) analysis indicated that ODN1-X underwent complete conversion to the new product ODN1-NRNO while ODN1-dU showed no reaction with NRNO, demonstrating that NRNO can just label ODN1-X (Fig. 1a and b). The NANO labelled DNA was identified by MALDI-TOF to ensure the reaction integrity (Fig. S4, ESI†). Moreover, the reaction products were analysed by denaturing polyacrylamide gel electrophoresis (PAGE) and imaged first by a Pharos FX Molecular imager (Bio-Rad, USA) (λ_{ex}: 532 nm) and then stained with Gel Red to observe the other DNA bands. Only in the ODN1-X with NRNO incubation can the formation of a new species directly excited with 532 nm be observed (Fig. 1c, lane 4). The new band after NRNO labelling showed a higher molecular weight than the other DNA substrate when stained with Gel Red dye (Fig. 1d). It was also noticed that the stripe of ODN1-X in lane 3 is lower than that of ODN1-dU in lane 1 because of the excision of dU. The selectivity of the fluorogenic labelling of UNG treated ODN1-dU can also be verified by the UV-vis spectra readout. The absorbance was detected at 585 nm, and the fluorescence emission maxima were found at 650 nm (Fig. 1e and Fig. S5, ESI†). Across the ODN series, almost no fluorescence at 650 nm was observed for NRNO, ODN1-dU, ODN1-dU + NRNO or ODN1-dU + UNG; but remarkable fluorescence enhancement was observed for ODN1-X + NRNO as a result of the blocking of the PET process (Fig. 1e). Then we investigated whether this reaction could be used in dsDNA. So, we chose a 15 bp dsDNA with dU as the model to react with NRNO under the same conditions and detected the fluorescence of the system. We found that the result is the same as for 15 nt ssDNA, for which an obvious fluorescence increase can also be observed after UNG treatment (Fig. S2, ESI†). To identify whether the fluorescence was only produced by the binding of the compound with a DNA major groove, NRNO was incubated with three 80 bp dsDNA with or without dU and no UNG treatment. No fluorescence increase could be observed, which means that the fluorescence "turn-on" is produced by the reaction of NRNO and the dU site after UNG excision (Fig. S3, ESI†).

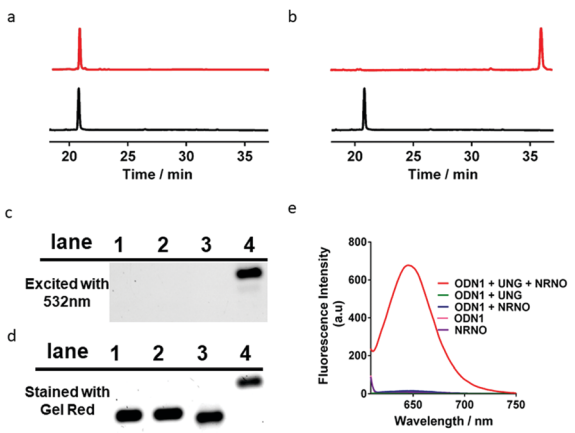

Fig. 1 (a) RP-HPLC traces of ODN1-dU (black) and ODN1-dU incubation with NRNO (red). (b) RP-HPLC traces of ODN1-X (black) and ODN1-X incubation with NRNO (red). (c and d) Denaturing PAGE analysis. Lane 1: ODN1-dU; lane 2: ODN1-dU after incubation with NRNO; lane 3: ODN1-X; lane 4: ODN1-X after incubation with NRNO. (e) Fluorescence emission spectra (λ_{ex}: 585 nm, λ_{em}: 650 nm) of ODN1-X after incubation with NRNO (red line) in comparison with other controls such as ODN1-X (green line), ODN1-dU after incubation with NRNO (blue line), ODN1-dU (pink line) and NRNO (purple line) under the same conditions.

Having established that NRNO shows high fluorescence selectivity toward ODN1-X, we next aimed to exploit this reactivity in the quantification of dU. Different concentrations of ODN1-dU were first incubated with UNG, then reacted with NRNO for fluorescence analysis (Fig. 2a). A linear correlation between the concentration of ODN1-dU and fluorescence intensity ranging from 50 nM to 1 M was also observed (Fig. 2b). Furthermore, a pool of mixtures with different contents of UNG treated ODN1-dU and untreated ODN1-dU moieties were reacted with NRNO for PAGE analysis. The increase in labelling bands was relative to the increasing amounts of UNG treated ODN1-dU both with 532 nm excitation and Gel Red staining (Fig. 2c). All of these results indicate that this is a highly selective and fluorescence-based switch-on method for the quantitative detection of dU.

After the reaction between abasic sites with the NRNO group, the bigger group of NRNO may have affected the binding of DNA polymerase. Thus, we supposed that the NRNO probe can not only be used for the quantitative detection of dU, but also for examining the status of dU modification at a particular position on the target DNA. To demonstrate the effect of NRNO in dU sites, we used ODN2-dU as a model test by primer extension assay. Firstly, UNG treated or untreated ODN2-dU were incubated with NRNO and the result was analysed by PAGE. Across the substrate series, similar to the results of ODN1-dU, we observed that NRNO could only label ODN2-dU treated with UNG, and showed no reaction with untreated ODN2-dU (Fig. S6, ESI†). The site-specific analysis of ODN2-dU

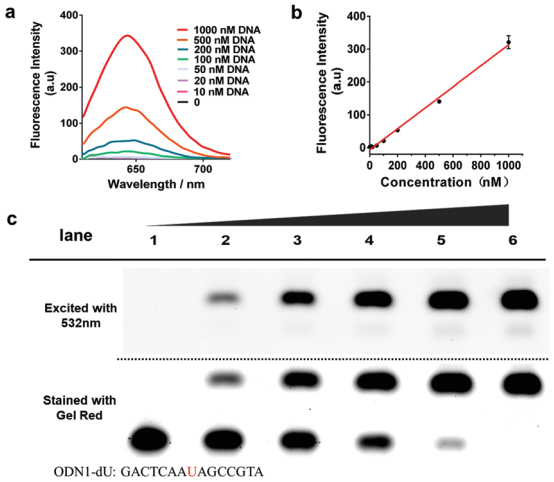

Fig. 2 (a) Fluorescence emission spectra (λ_{ex}: 585 nm, λ_{em}: 650 nm) of ODNs after reaction with NRNO. (b) Correlation of the fluorescence intensity (at 650 nm) of ODN1-X after fluorogenic labelling by NRNO with DNA concentration. (c) A mixture of ODN1-dU and ODN1-X after incubation with NRNO (increasing the ODN1-X content in the mixture from 0 to 100% with 20% increments).

in primer-extension assays showed that the NRNO-labelled nucleosides may act as a "roadblock" to abort the primer extension by the Bst DNA polymerase in a reaction time of 30 min and enable the detection of the site information of dU (Fig. 3). We observed that only the ODN2-dU in lanes 2 and 3 without UNG treatment can achieve a complete extension product. The NRNO labelled ODN2 in lane 5 exhibited significant extension stalled products before the dU site. Besides, the main extension product of UNG treated ODN2-dU in lane 4 appears to only extend by one base in the X site which is consistent with the result of a previous report.

Next, we applied this assay to detect dU in genomic DNA. Total genomic DNA HEK293T cells were isolated with a commercially available kit, and then treated by UNG, following incubation with NRNO for fluorometric analysis. The total RNA without UNG treatment was used as negative control. As the result indicated, the UNG treated DNA showed a significantly higher signal compared with untreated DNA, which indicated the existence of dU in genomic DNA (Fig. 4a). And the fluorescence

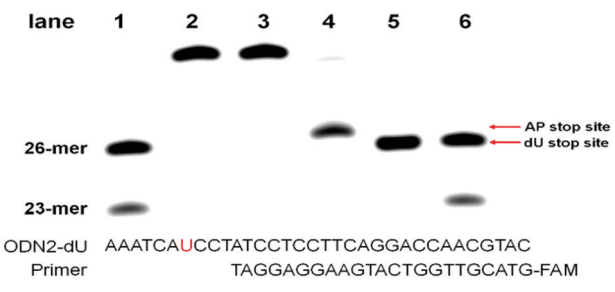

Fig. 3 Primer-extension assay with Bst DNA polymerase. Lane 1 and lane 6: marker; lane 2: ODN2-dU; lane 3: ODN2-dU after incubation with NRNO; lane 4: ODN2-X (ODN2-dU after treatment with UNG); lane 5: ODN2-NRNO (ODN2-X after incubation with NRNO).

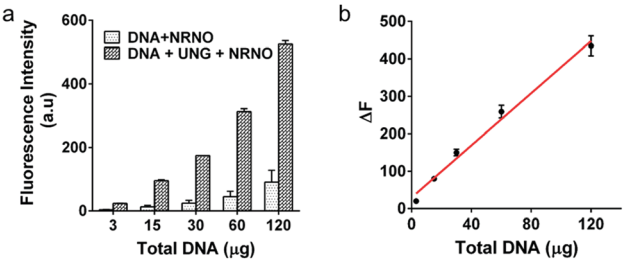

Fig. 4 (a) Fluorescence intensity (λ_{ex}: 585 nm, λ_{em}: 650 nm) of total DNA from 293T cells after treatment with UDG and incubation with NRNO. Total RNA incubated only with NRNO was used as a control. (b) Correlation of the fluorescence changes (at 650 nm) with different DNA contents.

of untreated DNA was produced by the natural abasic sites in 293T cells.[24] In addition, the fluorescence increased in a dose-dependent manner, which further certified the feasibility of this strategy (Fig. 4b). We also observed the lower signal of untreated DNA after NRNO incubation which may be induced by the pre-existing X sites in genomic DNA. It also indicated the potential application of NRNO for X site detection in genomic DNA.

NRNO was reported as a far-red emissive, two-photon excitable probe which endows advantages for bio-imaging. We proceeded to evaluate the ability of the two-photon action cross sections ($\phi\delta$) of this probe before and after incubation with UND treated DNA oligo containing dU, which are crucial parameters for two-photon microscopy. Similar to a low quantum yield, the NRNO probe itself possessed an extremely low TP action cross section, which was undetectable under our experimental conditions. In contrast, the reaction product of NRNO with UDN treated ODN-dU showed the maximal TP action cross section value of 72 GM (1 GM = 10–50 cm^4 s per photon per molecule) at 710 nm (Table S1 and Fig. S7, ESI†). This result indicates that the probe shows a distinct change in TP fluorescence properties before and after reaction with the target, which implies that NRNO is suitable for tracking dU with the help of UNG under TP excitation for cell imaging.

To explore this hypothesis, we used the UNG2 overexpressed HEK293T cells which were developed in a previous study.[16] The cytotoxicity of NRNO was first evaluated by MTT assays with HEK293T cells. No cellular toxicity was observed with 24 h of incubation of up to 25 μM NRNO demonstrating the low cytotoxicity of the probe (Fig. S8, ESI†). Overexpressed UNG2 and wild-type HEK293T cells were incubated with 5 μM NRNO for 12 h, then analysed by a TP mode confocal laser scanning microscope (λ_{em} = 710 nm) after washing the cells with PBS (0.01 M) three times. As demonstrated in Fig. S9a and b (ESI†), the red fluorescence of UNG2 overexpressed cells was significantly enhanced (1.7-fold, as quantitatively calculated and presented in Fig. S9c, ESI†) compared to the wild type. These results confirm the capability of NRNO to detect dU in cells. To verify this result, total DNA extracted from wild-type and UNG2 overexpressed HEK293t cells was incubated with NRNO, then analysed by fluorescence. As the result indicated, total DNA from UNG2 overexpressed HEK293T cells showed a higher fluorescence signal consistent with cell imaging (Fig. S10, ESI†).

In conclusion, we have presented a far-red emissive, two-photon NRNO probe with an adequate TP cross section and a far-red emission band centred at 650 nm which can selectively label dU with treatment by UNG. Given the rapidly expanding interest in the role of dU in biological functions, this probe offers a reliable, fast, cheap and easy method to gain quantitative and qualitative information about uracil levels in DNA. Furthermore, this simple assay can also be applied to the analysis of X sites in genomic DNA and provides a reference and tool for future investigations of deoxyuridine modification.

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Conflicts of interest

There are no conflicts to declare.

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