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## Fluorescent Wittig reagent as a novel ratiometric probe for the quantification of 5-formyluracil and its application in cell imaging†

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**The chemically selective detection of natural nucleobase modifications has been regarded as the key step in understanding their important roles in epigenetics. Herein, for the first time, we introduce a Wittig reaction into the design of reaction-based fluorescent probes for ratiometrically detecting 5fU, selectively labelling 5fU-modified DNA and imaging intracellular 5fU produced by  $\gamma$ -irradiation.**

As natural nucleobase modifications produced by oxidation of thymine and cytosine, 5-formyluracil (5fU) and 5-formylcytosine (5fC) play important roles in epigenetics.<sup>1</sup> Recent discoveries have indicated that 5fU and 5fC are highly involved in DNA methylation and demethylation processes.<sup>2</sup> They are also regarded as oxidative damage<sup>3</sup> which results in gene regulation by causing genotoxic lesions, introducing base mispairing and inhibiting sequence-specific DNA–protein interactions.<sup>4</sup> Compared with 5fC, 5fU is more prone to exist in the ionized form, which is likely responsible for the high mutagenesis frequency of 5fU during DNA replication under physiological conditions both *in vitro* and *in vivo*.<sup>5</sup> Recently, Balasubramanian *et al.* revealed that it is 5fU in synthetic oligodeoxynucleotides that alters DNA structures.<sup>6</sup> Therefore, developing methods to sensitively and selectively detect formyl pyrimidines can not only help to understand their epigenetic roles, but can also provide a detection technique for studying the relationship between formyl pyrimidines and diseases. In particular, quantitative analysis methods for 5fU in DNA with high accuracy are urgently required.

To date, HPLC-MS together with complete enzymatic digestion of target DNA has been well established as a powerful tool for 5fU/5fC detection, although it is time-consuming, expensive and requires an isotope-labelled internal standard.<sup>7</sup> In the last ten years, focus has been on the highly reactive 5-formyl group, and *O*-amino(thio)phenol,<sup>8</sup> amine,<sup>9</sup> hydroxylamine,<sup>10</sup> hydrazide,<sup>11</sup>

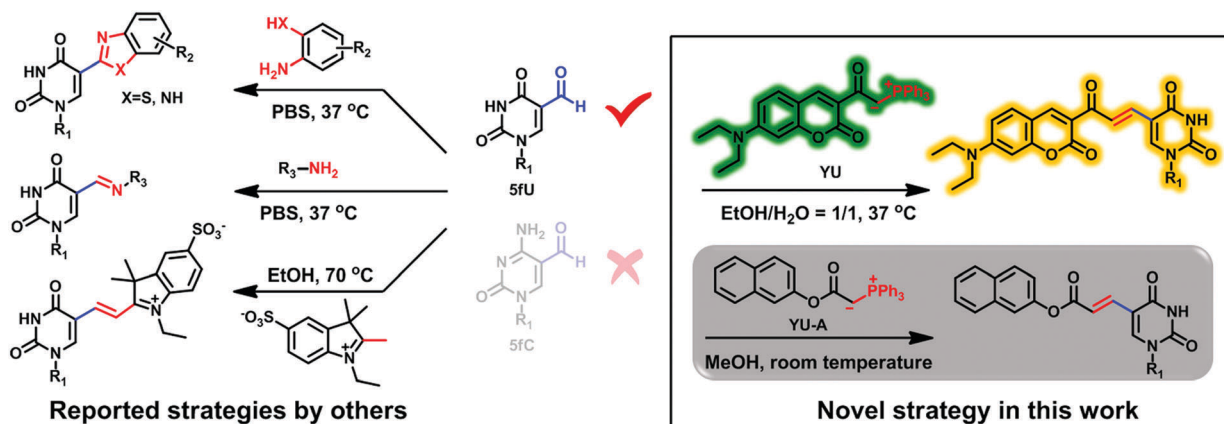
indole,<sup>12</sup> benzothiazole<sup>13</sup> and 1,2-phenylenediamine<sup>14</sup> derivatives for applications as potential fluorescent labels or small molecule tags to attach to 5fU or/and 5fC-modified DNA for detection, sequencing and enrichment.<sup>15</sup> Taking advantage of widely used fluorescent probe technology and chemoselective reactions, these original studies have provided effective platforms for biological events analysis. However, it should be pointed out that their quantitation mechanism primarily involves a Schiff base reaction or Aldol-type (Knoevenagel-like) condensation, although their probes vary in structure. Therefore, the development of new aldehyde-reagents based on other organic chemistry principles to expand the library of fluorescent probes toward 5fU is of great significance and necessity.

In the past few years, we have gained a lot of experience in designing reaction-based probes for reactive oxygen/sulfur species as well as others.<sup>16</sup> Herein, our intention is to construct conjugated C=C bonds by introducing new chemical reactions. The Wittig reaction,<sup>17</sup> discovered by Georg Wittig in 1953 and winner of the Nobel prize in 1979, is a general methodology for synthesizing alkenes from aldehydes or ketones. Given the outstanding contributions of Wittig olefination in organic synthesis, a few scientists have used Wittig reagents to modify proteins bearing an “aldehyde tag” in recent years,<sup>18</sup> and we will take on the challenge of its application in nucleic acid labelling, which has never been reported so far.

In this work, we have proved the feasibility of the Wittig reaction in detecting nucleoside modifications. As outlined in Scheme 1, a coumarin scaffold was selected as the fluorophore owing to its good photostability, high quantum yield and large Stokes shift. Furthermore, the lactone structure coupled with a 7-carbonyl group can stabilize the adjacent carbanion, making it easy to preserve and avoiding anhydrous/anaerobic environments when reacting with aldehydes. Our elaborately designed fluorescent Wittig reagent **Ylide U** (abbr. as **YU**), could selectively tag 5fU in DNA *via* a Wittig reaction, while 5fC and the abasic sites (AP) weren't disturbed. After the reaction, **YU-5fU** (Scheme S3 and Fig. S3, ESI†) with an emission peak at 555 nm was obtained in good yield. In addition, we also designed **YU-A**, **YU-B** and **YU-C**

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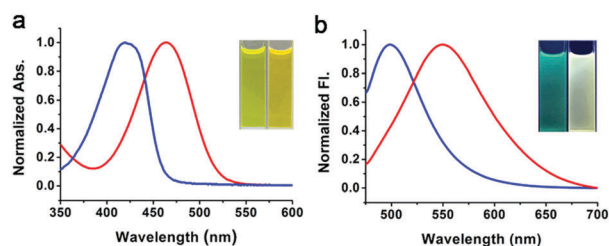
**Scheme 1** The reported strategy for fluorogenic labelling of 5-formyluracil and the novel strategy for fluorogenic labelling of 5-formyluracil in DNA based on the Wittig reaction.

(Schemes S6 and S7, ESI<sup>†</sup>), in which naphthalene, coumarin and tetraphenylethylene (TPE) served as fluorophores, respectively. However, we failed to synthesise **YU-B** and **YU-C** due to the instability of the phenol ester groups in their structures. Although **YU-A** was successfully obtained, it partially decomposed during alkalization before reacting with **5fU**. **YU** was synthesized readily in four steps using 4-(diethylamino)salicylaldehyde and ethyl acetoacetate as the starting materials (Scheme S1, ESI<sup>†</sup>). The structure of **YU** was fully characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR, and HRMS (see ESI<sup>†</sup>).

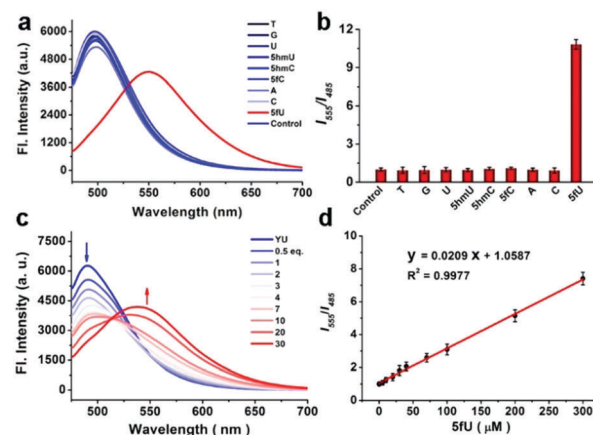
To explore the feasibility of our design, we first studied the Wittig reactivity between **YU** and the **5fU** nucleoside in CH<sub>3</sub>OH (Scheme S3, ESI<sup>†</sup>). As expected, after incubation at 37 °C for 24 h, an orange product which proved to be a **YU-5fU** adduct was obtained in more than 85% yield. Then, we measured the UV absorbance and fluorescence emission properties of **YU** and **YU-5fU** in various solvents. As shown in Fig. S1, and Tables S1 and S2 (ESI<sup>†</sup>), different degrees of red shift were observed in both absorption and emission spectra, which is consistent with our theoretical prediction and also indicates that **YU** could be applied as a ratiometric fluorescent probe for the recognition of **5fU**. The detection reaction toward **5fU** could proceed smoothly in pure water or under weak acid/base conditions (Fig. S2, ESI<sup>†</sup>). All of these results prompted us to optimize our experimental conditions such as solvent, temperature, reaction time, *etc.* Finally, the detection was carried out in EtOH/H<sub>2</sub>O (1/1, v/v) at 37 °C for 48 h. As can be seen from Fig. 1, before the reaction, **YU**

exhibited an absorption maximum at 411 nm and an emission maximum at 498 nm ( $\Phi = 0.5\%$  in DMSO), respectively. Upon addition of **5fU**, the absorption intensity at 411 nm decreased and a redshift of 57 nm, as well as a deeper colour, was observed. Concomitantly, the characteristic emission band belonging to **YU** vanished completely, while an intense fluorescence emission band centred at 555 nm appeared, accompanied with a green-to-yellow solution fluorescence colour change. Notably, the spectral performance of the reaction mixture is in good agreement with pure **YU-5fU** ( $\Phi = 9.5\%$  in DMSO, see Fig. S3, ESI<sup>†</sup>), indicating that the yield of this Wittig detection was really high.

We then evaluated the specificity of **YU** for **5fU** compared to various potential interfering species, including five canonical deoxynucleosides and their natural modifications, named 5hmC, 5hmU, and **5fC** under the optimized conditions. The data (Fig. 2a and b) show that other deoxynucleosides induced no obvious spectral changes for **YU**, and only **5fU** triggered a remarkable enhancement of the emission ratio  $I_{555}/I_{485}$ . At the



**Fig. 1** (a) Normalized UV/vis absorption spectra and (b) fluorescence emission spectra ( $\lambda_{\text{ex}}$ : 460 nm) of **YU** before (blue) and after (red) reaction with **5fU** in EtOH/H<sub>2</sub>O medium (1/1, v/v) at 37 °C for 48 h. Inset: Images of **YU** before and after reaction with **5fU** under daylight or UV lamp (365 nm).



**Fig. 2** (a) Fluorescence spectra and (b) the emission intensity ratio ( $I_{555}/I_{485}$ ) of **YU** after being incubated with **5fU** and other interfering species, including A, G, C, T, U, 5hmU, 5hmC and **5fC**. 10  $\mu\text{M}$  **YU** without any nucleoside serving as the control. (c) Fluorescence titration spectra and (d) linear relationship of the emission intensity ratio ( $I_{555}/I_{485}$ ) of **YU** (10  $\mu\text{M}$ ) toward **5fU** (0–300  $\mu\text{M}$ ).  $\lambda_{\text{ex}}$ : 460 nm.

same time, only the reaction liquid corresponding to **5fU** undergoes a colour change (Fig. S4, ESI<sup>†</sup>), which might be ascribed to its active aldehyde group and the highly selective Wittig reaction. In 2011, Thomas Carell *et al.* first reported the crystal structure of **5fC**, and revealed a strong intramolecular H-bond between the exocyclic amino group NH<sub>2</sub>(4) and the carbonyl-oxygen at C5,<sup>19</sup> which was suggested to be the molecular reason why **5fC** is less reactive than **5fU** and why our Wittig reagent can react with **5fU** rather than **5fC**.

Subsequently, fluorescence titration experiments of **YU** toward **5fU** were conducted. As depicted in Fig. 2c and d, with an increase of **5fU** (0–300 μM), the fluorescence emission band centred at 485 nm drops gradually and a distinct rise at 555 nm occurs simultaneously, indicating a **5fU**-mediated transformation from **YU** to **YU-5fU**. The detection limit of **YU** toward **5fU** was calculated to be 0.33 μM. These results verify that **YU** can sensitively detect **5fU** under mild conditions.

Encouraged by the above-mentioned findings, we further estimated the ability of **YU** to label **5fU**-modified DNA. Generally, we chose an oligodeoxynucleotide containing one **5fU** (ODN-**5fU**) as a model and incubated it with a large excess of **YU** (50 eq.) to ensure complete DNA labelling under optimized conditions. Considering that ODN-**5fC** (where the **5fU** site is replaced by **5fC**), ODN-AP (contains one abasic site bearing an aldehyde source), ODN-C, and ODN-T (where the **5fU** site is C or T) might affect the Wittig reaction between **YU** and ODN-**5fU**, the fluorescence responses of **YU** toward these interfering species were tested at the same time. The data (Fig. 3a and Fig. S5, ESI<sup>†</sup>) demonstrate that only **5fU** showed an 8-fold fluorescence enhancement at 555 nm, other ODNs, including ODN-**5fC** and ODN-AP, showed negligible spectral fluctuation on **YU**. The superior selectivity was attributed to the higher reactivity of ODN-**5fU** and the specificity of the Wittig reagent.

We further attempted a denaturing polyacrylamide gel electrophoresis (PAGE) experiment. Individually, ODN-**5fC**, ODN-AP, ODN-T, and ODN-C were incubated with **YU** in EtOH/H<sub>2</sub>O (1/1, v/v) at 37 °C. 48 h later, the reaction mixtures were directly mixed with a loading buffer and then underwent electrophoresis without further purification. Once a fluorophore was introduced into DNA, a fluorescent band was visualized on the gel imaging system. As shown in Fig. 3b, we indeed observed a distinct fluorescent band in lane 5, corresponding to the reaction product derived from ODN-**5fU**, while no signal was found in

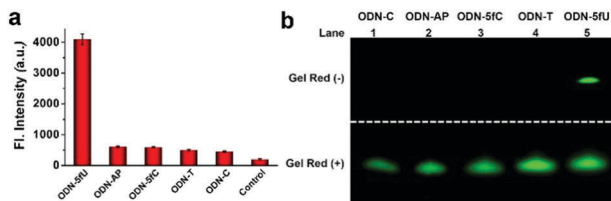


Fig. 3 (a) Fluorescence intensity of **YU** at 555 nm after reaction with ODN-**5fU**, ODN-**5fC**, ODN-AP, ODN-T and ODN-C. 10 μM **YU** without any ODN served as the control.  $\lambda_{\text{ex}}$ : 495 nm. (b) PAGE analysis of ODN-**5fU**, ODN-**5fC**, ODN-AP, ODN-T and ODN-C after incubation with **YU**. The images before and after SuperRed staining are above and below the dashed line, respectively.

the control lanes. The gel was then stained with 3× SuperRed to image the remaining DNA bands. The PAGE experiment confirmed the ability of **YU** to distinguish ODN-**5fU** from other DNA modifications once again.

Given that **YU** shows an excellent performance in detecting **5fU** in buffer, we proceed to investigate whether it is capable of imaging **5fU** in HeLa cells. Pouget<sup>20</sup> and Wang *et al.*<sup>7a</sup> have reported that  $\gamma$ -rays would increase **5fU** mutations in some mammalian cells. We hence exposed HeLa cells to a <sup>60</sup>Co  $\gamma$ -source at a dose rate of 18 Gy min<sup>−1</sup> (60 min, r.t.).<sup>14b</sup> Immediately after irradiation, the cells were fixed and incubated with **YU** at 37 °C. As can be seen from Fig. 4a–i, upon excitation at 488 nm,  $\gamma$ -irradiated HeLa cells, generating much more **5fU**, displayed bright fluorescence in the emission range of 500–600 nm, whereas those without  $\gamma$ -irradiation maintained weak fluorescence. Fig. 4j shows that there was almost 1-fold enhancement in the average fluorescence intensities caused by  $\gamma$ -exposure or increased **5fU**. In fact, the bulk of DNA damage induced by the  $\gamma$ -source is mediated by hydroxyl radicals ( $\cdot\text{OH}$ ), which act as an indirect effect of water radiolysis.<sup>21</sup> In view of this, we pre-treated HeLa cells with Fenton's reagent (FeSO<sub>4</sub>:H<sub>2</sub>O<sub>2</sub> = 1:5) and then incubated them with **YU** for different times. Confocal microscopy analysis reveals that the fluorescence became stronger as the incubation time increased (Fig. S6a, ESI<sup>†</sup>). At the same time, the cells pre-treated with **5fU** before incubation with **YU** exhibit the same performance (Fig. S6b, ESI<sup>†</sup>). Furthermore, we also treated HeLa cells with different doses of  $\cdot\text{OH}$  to stimulate the cells to produce different doses of **5fU** mutations. As shown in Fig. S7a (ESI<sup>†</sup>), a progressively brighter fluorescence was observed. However, compared to 25 μM  $\cdot\text{OH}$ -stimulated cells, 50 μM  $\cdot\text{OH}$ -stimulated cells did not exhibit significant fluorescence enhancement, probably because 25 μM  $\cdot\text{OH}$  caused saturation to be reached, or the cell began to repair the **5fU** lesion more extensively over such a long incubation period. This may also be the reason why the fluorescence intensity is consistent even if the cells are pre-treated with different concentrations of **5fU** (Fig. S7b, ESI<sup>†</sup>). All of the above results imply that **YU** could respond to intracellular **5fU**.

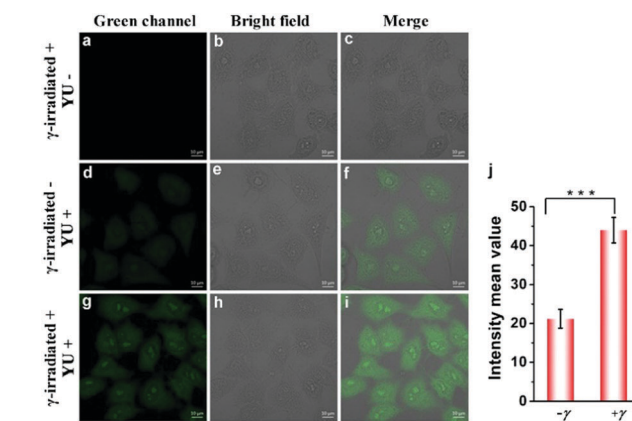


Fig. 4 (a–i) Confocal fluorescence images of  $\gamma$ -irradiated HeLa cells with **YU** (10 μM). (j) Statistical analysis (average fluorescent intensity calculated from d and g) was performed using Student's *t*-test ( $n$  = 10 fields of cells). \*\*\* $P$  < 0.001, error bars are  $\pm$ SEM. Scale bar: 10 μm. The images were acquired upon excitation at 488 nm and fluorescence emission was collected at 500–600 nm.

In summary, we have established a novel strategy to design and synthesize a small molecular fluorescent probe based on the Wittig reaction, for ratiometrically detecting 5fU, labelling 5fU-modified DNA and imaging intracellular 5fU. The sensitivity and selectivity of a coumarin-derived phosphorus ylide toward an active aldehyde were confirmed by our studies. These results broaden the application of the Wittig reaction and offer an attractive candidate for marking aldehyde-containing biologically related species.

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## Conflicts of interest

There are no conflicts to declare.

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