



# "Click" disaggregation-induced emission of a fluorescent dye†

Kaleena Basran<sup>a</sup> and Nathan W. Luedtke<sup>id</sup> \*<sup>ab</sup>

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Here we demonstrate a new approach to fluorogenic labelling, where a cationic hemicyanine (CHyC) exhibits disaggregation-induced emission (DIE) upon undergoing an azide–alkyne "click" reaction. CHyC self-associates and is self-quenched in aqueous buffer over a low micromolar concentration range. When an azido nucleoside (AmdU) or azide-containing cellular DNA is added to CHyC in the presence of Cu(I), a copper-catalysed azide–alkyne cycloaddition drives dye disaggregation, significantly increasing the fluorescence intensity of the probe upon its covalent attachment to modified biomolecules.

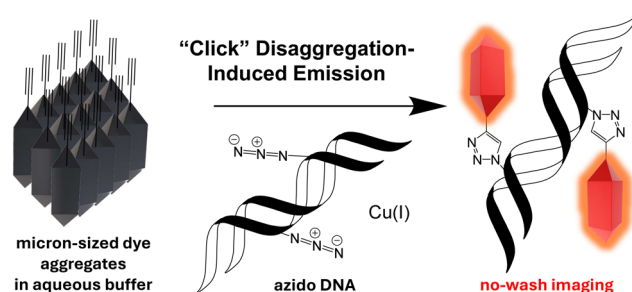
Fluorogenic bioorthogonal "click" chemical reactions can enable convenient, no-wash cellular imaging.<sup>1</sup> In the context of nucleic acids,<sup>2</sup> click reactions with fluorescent probes provide powerful tools for characterizing DNA/RNA metabolism, cell cycle progression, viral entry, and therapeutic mechanisms of known and new drug candidates.<sup>3</sup> Classical fluorophores like rhodamines, cyanines, coumarins, and others<sup>4</sup> are now widely available with clickable handles—such as tetrazines, azides, and alkynes—to facilitate conjugation reactions such as copper-catalysed azide–alkyne cycloadditions (CuAAC).<sup>5</sup> Increasing the fluorescence intensity of the labelled biomolecule as compared to the unreacted dye is an important and challenging goal in wash-free imaging applications.<sup>6</sup>

Cyanine dyes are a diverse family of fluorophores which are classified by the number of methine "bridge" units and terminal heterocycles present.<sup>7</sup> Styryl hemicyanines containing two methine carbons have been used in three-way junction DNA aptamers,<sup>8</sup> fluorescent oligonucleotide probes,<sup>9</sup> and for non-covalent binding of DNA.<sup>10</sup> Moreover, the metabolic modification of nucleic acids with alkene groups followed by treatment with tetrazine-substituted styryl hemicyanines enabled inverse electron-demand Diels–Alder (IEDDA) reactions on cellular

DNA.<sup>11</sup> Indeed, tetrazines are well established to quench fluorophores,<sup>12</sup> allowing for wash-free imaging of metabolically labelled DNA in live cells.<sup>6b</sup>

Azides groups are invaluable in chemical biology and drug development due to their small size and bioorthogonal reactivity.<sup>13</sup> Despite their widespread applications,<sup>3f,14</sup> a general "turn-on" strategy for azide-reactive dyes remains elusive. Azide–alkyne cycloadditions are not inherently fluorogenic, although triazole formation has been shown to result in increased in emissions of highly tailored systems.<sup>15</sup> Exploring innovative turn-on mechanisms for azide-modified nucleic acids, such as disaggregation-induced emission (DIE) where fluorescence is triggered by the disaggregation of aggregated probes is a promising new approach (Scheme 1).<sup>16</sup> Non-covalent DIE reactions have previously been used for detecting small molecules,<sup>17</sup> monitoring the equilibrium of G-quadruplexes,<sup>18</sup> and probing cellular membranes and proteins.<sup>19</sup> Herein, we designed a cationic hemicyanine (CHyC) that exhibits DIE upon reacting with an azide-containing nucleoside, 5-(azidomethyl)-2'-deoxyuridine (AmdU),<sup>14d</sup> via CuAAC reaction. The irreversible covalent chemical reaction shifts the dye self-association equilibrium towards disaggregation, resulting in enhanced fluorescence emission.

To synthesize CHyC, 6-methoxy-2-naphthaldehyde **1** was transformed into benzoindole **2** through a base-promoted



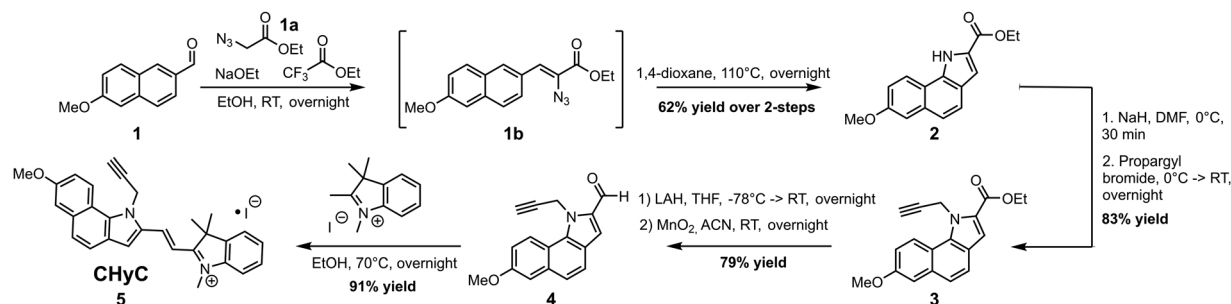
**Scheme 1** A quenched and aggregated alkyne-containing fluorescent dye undergoes disaggregation and enhanced fluorescence upon CuAAC reaction with azido DNA.

<sup>a</sup> Department of Chemistry, McGill University, Montreal, Quebec H3A 0B8, Canada

<sup>b</sup> Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6, Canada

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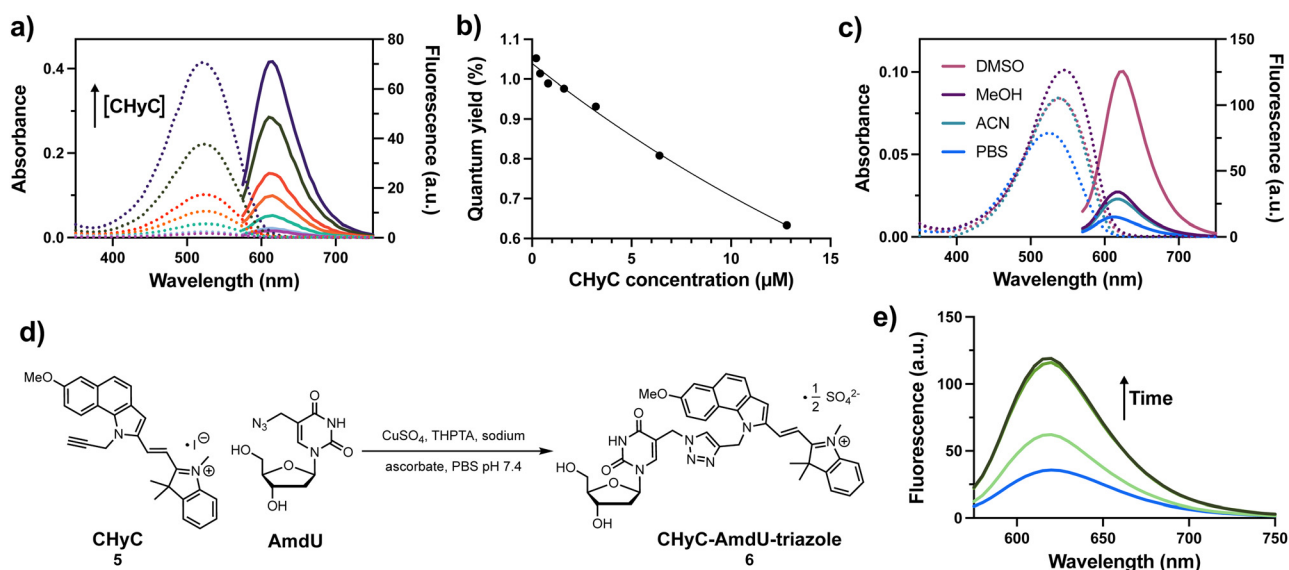


**Scheme 2** Synthesis of CHyC (**5**) and all relevant intermediates where EtOH = ethanol, DMF = *N,N*-dimethylformamide, LAH = lithium aluminium hydride, THF = tetrahydrofuran, and ACN = acetonitrile. See the ESI† for the synthesis and characterization of these compounds.

Knoevenagel condensation and Hemetsberger indolization (Scheme 2).<sup>20</sup> First, ethyl-2-azidoacetate **1a** was synthesized in a 98% yield from ethyl-2-bromoacetate.<sup>21</sup> 6-Methoxy-2-naphthaldehyde **1** and azidoacetate **1a** were dissolved in ethanol along with a sacrificial electrophile, ethyl trifluoroacetate. 20% sodium ethoxide in ethanol was added at 0 °C and the reaction was stirred overnight yielding the  $\alpha$ -azido- $\beta$ -arylacrylate **1b**. Thermolysis of intermediate **1b** gave the benzo[*g*]indole **2** as the only regioisomeric indole in a 62% yield over two-steps. The propargyl group was introduced by treating **2** with sodium hydride followed by the dropwise addition of propargyl bromide to give the desired product **3** in an 83% yield. **3** was then reduced to the corresponding aldehyde **4** in two consecutive steps in a 79% yield. **4** and 1,2,3,3-tetramethyl-3*H*-indol-1-ium iodide were heated to 70 °C overnight in ethanol to yield CHyC **5** as a dark purple solid with low water solubility in 91% isolated yield (Scheme 2). The probe and all relevant intermediates were fully characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR, and high-resolution ESI MS (see ESI†). Stock solutions of CHyC for

photophysical and biological studies were prepared in DMSO and diluted into the indicated solvents (0.5% DMSO unless stated otherwise) prior to analysis.

The photophysical properties of CHyC **5** were evaluated at various concentrations upon dilution into 1× PBS buffer, pH = 7.4 (Fig. 1a). The aqueous samples displayed a linear relationship between absorbance ( $\lambda_{\text{max}} = 520$  nm) and CHyC concentration over the range 0.2–12.4  $\mu\text{M}$  (ESI,† Fig. S1,  $\epsilon_{520} = 32\,300\text{ cm}^{-1}\text{ M}^{-1}$ ). In contrast, non-linear concentration-dependent effects were observed in the fluorescence emission intensities of the same samples (ESI,† Fig. S1), giving lower quantum yield values ( $\Phi = 1.1\text{--}0.063\%$ ) with increasing concentration (Fig. 1b). Microscopic evaluation of the samples prepared at 2–10  $\mu\text{M}$  in PBS revealed the presence of purple, non-fluorescent particles with diameters ranging from roughly 2–8  $\mu\text{m}$  (ESI,† Fig. S2). In contrast, CHyC samples prepared entirely in DMSO exhibited better solubility, a higher measured extinction coefficient ( $\epsilon_{545} = 41\,900\text{ cm}^{-1}\text{ M}^{-1}$ ) and concentration-independent quantum yield ( $\Phi = 5.4\%$ ). In DMSO, CHyC



**Fig. 1** (a) Absorbance (dashed) and fluorescence (solid) spectra of 0.2–12.4  $\mu\text{M}$  solutions of CHyC **5** in 1× PBS (pH 7.4, 2% EtOH). (b) Calculated quantum yields versus CHyC concentrations in 1× PBS. (c) Absorbance (dashed) and fluorescence (solid) spectra of a 2  $\mu\text{M}$  solution of CHyC **5** in various solvents and 1× PBS (pH 7.4, 2% EtOH). (d) CuAAC reaction of CHyC **5** and AmdU where THPTA = tris(benzyltriazolylmethyl)amine. (e) Fluorescence spectrum of a 100  $\mu\text{M}$  solution of CHyC, 1 mM  $\text{CuSO}_4$ , 2 mM THPTA, 1 mM AmdU, and 10 mM sodium ascorbate in PBS pH 7.4 (1.3% DMSO) at time = 0 min, 20 min, 40 min, and 60 min into the reaction. For all fluorescence: ex: 546 nm, em: 570–750 nm. See the ESI† for the characterization of CHyC–AmdU–triazole **6**.

exhibited a red-shifted absorbance ( $\lambda_{\text{max}} = 540 \text{ nm}$ ) and emission ( $\lambda_{\text{max}} = 625 \text{ nm}$ ) as compared to  $1 \times \text{PBS}$ . The absorbance spectrum of CHyC in acetonitrile (ACN) closely resembled that of DMSO. However, in methanol (MeOH), additional solvent effects led to a further redshift of CHyC, albeit with a lower quantum yield ( $\Phi = 1.0\%$ ) than the  $5.4\%$  for DMSO (Fig. 1c and ESI,† Table S1). Together these results suggest that the micro-aggregated form(s) of CHyC in PBS have some twisting about the styryl bridge and/or self-assembly into H-type aggregates.<sup>22</sup> The dynamic, self-quenching and self-association behaviour of CHyC over the low  $\mu\text{M}$  concentration range suggested that it may exhibit “turn-on” fluorescence behaviour upon chemical reaction with groups that would endow enhanced solubility properties of the product in water.

To evaluate if a click reaction involving a partially soluble dye can induce disaggregation-induced emission (DIE), a  $100 \mu\text{M}$  solution of CHyC **5** was subjected to standard CuAAC conditions with a 10-fold excess of AmdU in  $1 \times \text{PBS}$  containing  $1\%$  DMSO (Fig. 1d). The reaction was monitored by fluorescence (Fig. 1e) as well as high performance liquid chromatography (ESI,† Fig. S3). Both analyses indicated complete consumption of CHyC **5** in less than one hour. Remarkably, the fluorescence intensity of the solution showed a  $\sim 3$ -fold increase; reminiscent of the changes observed in DMSO (Fig. 1c). The CHyC-AmdU-triazole reaction product **6** was isolated in a  $70\%$  yield and was characterized to confirm its identity (see ESI†). These results demonstrate that DIE during a bioorthogonal chemical reaction can be used to track reaction progress in real time.

To evaluate the potential utility of DIE of CHyC in no-wash cellular staining and imaging, HeLa cell cultures were treated with  $100 \mu\text{M}$  of an AmdU monophosphate derivative bearing two 5'-pivaloyloxymethyl masking groups “POM-AmdU”,<sup>23</sup> for 17 hours prior to fixation and staining with  $10 \mu\text{M}$  CHyC in  $1 \times \text{PBS}$  containing  $1\%$  DMSO and  $\text{Cu(I)}$ . The cells were imaged while still in the staining solution, revealing large fluorescence enhancements of the nuclei in cells pre-treated with POM-AmdU as compared to those receiving vehicle only. As a control, we compared the performance of CHyC with a commercially available Cy5 alkyne derivative “Alexa Fluor™ 647 Alkyne” that was also found to be compatible with no-wash imaging, but it displayed little or no selectivity for the cellular nuclei of cells that had been pre-treated with POM-AmdU (ESI,† Fig. S4). To evaluate the DNA selectivity of CHyC staining in POM-AmdU treated cells, the CHyC staining solutions were removed by aspiration, and a second solution containing the non-covalent DNA stain Hoechst 33342 was added to the cells and imaged without washing (Fig. 2). Only cells receiving POM-AmdU exhibited CHyC “turn-on” fluorescence that co-localized with Hoechst staining with a Pearson correlation coefficient (PCC) of  $0.76 \pm 0.03$  as compared to a  $\text{PCC} = 0.31 \pm 0.08$  for the control cells not pre-treated with POM. A perfect correlation of  $1.0$  was not expected because only a fraction of the cells had passed through S-phase during the 17-hour incubation with POM-AmdU.

In summary, CHyC is a novel cationic hemicyanine dye that undergoes disaggregation-induced emission (DIE) after CuAAC click reactions. In the current example, DNA is targeted by

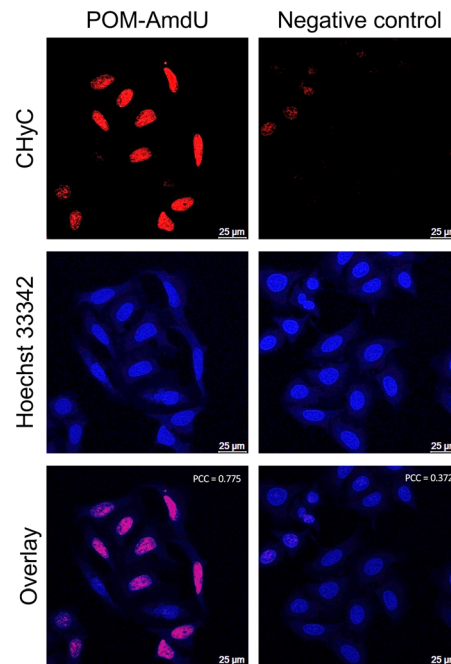


Fig. 2 Visualization of azide-modified DNA in HeLa cells treated with  $100 \mu\text{M}$  of POM-AmdU for 17 hours followed by fixation and no-wash CuAAC staining with  $10 \mu\text{M}$  CHyC **5** in the presence of  $1 \text{ mM}$   $\text{CuSO}_4$ ,  $2 \text{ mM}$  THPTA, and  $10 \text{ mM}$  sodium ascorbate for 2 hours. The CuAAC solution was aspirated without washing, and Hoechst 33342 was used added as nuclear co-stain and directly imaged. Negative control samples received no POM-AmdU but were otherwise treated identically.

virtue of AmdU incorporation into cellular DNA. In theory, RNA could be targeted by CHyC by using appropriate metabolic labels such as  $N^6$ -ethylazido-adenosine or  $2'$ -azidoadenosine.<sup>14f</sup> While fast, the CuAAC reaction is limited to fixed cells due to its toxicity,<sup>24</sup> and hence catalyst-free DIE reactions based on SPAAC<sup>25</sup> or vinyl-tetrazine ligation<sup>6b</sup> could provide future access to wash-free imaging of live cells.

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## Data availability

The data supporting this article have been included in the main article and as part of the ESI.†

## Conflicts of interest

There are no conflicts to declare.

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