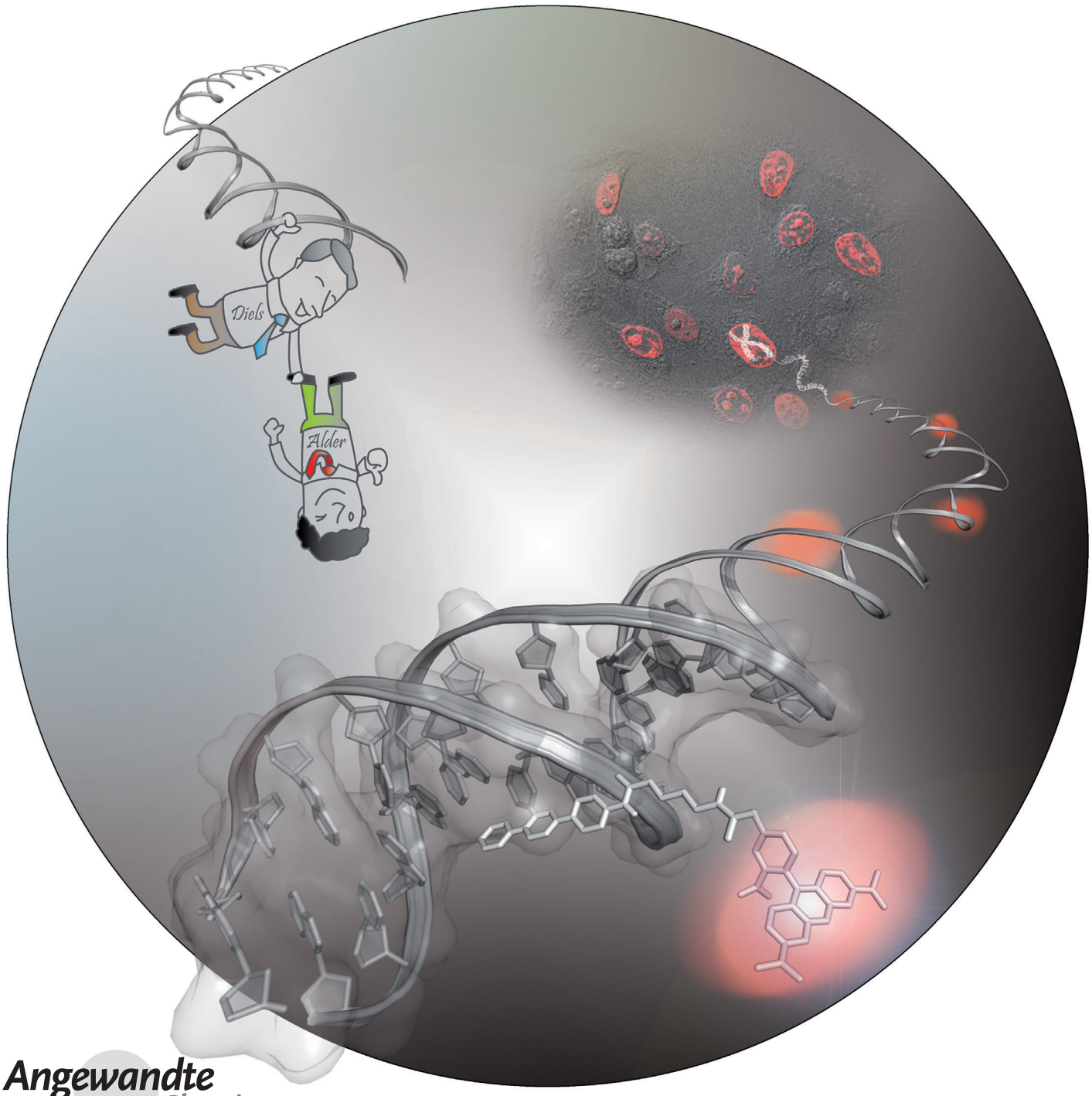
Angewandte Zuschriften

VIP DNA Labeling Very Important Paper

DOI: 10.1002/ange.201403580

Alkene–Tetrazine Ligation for Imaging Cellular DNA**

*Ulrike Rieder and Nathan W. Luedtke**



Angewandte Chemie

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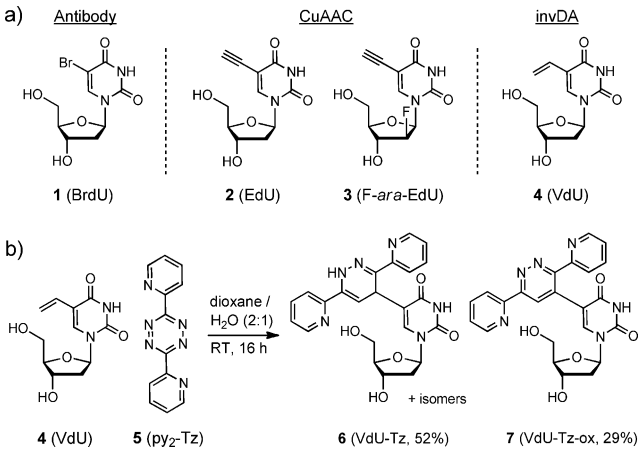
Abstract: *5-Vinyl-2′-deoxyuridine (VdU) is the first reported metabolic probe for cellular DNA synthesis that can be visualized by using an inverse electron demand Diels–Alder reaction with a fluorescent tetrazine. VdU is incorporated by endogenous enzymes into the genomes of replicating cells, where it exhibits reduced genotoxicity compared to 5-ethynyl-2′-deoxyuridine (EdU). The VdU–tetrazine ligation reaction is rapid ($k \approx 0.02\,M^{-1}s^{-1}$) and chemically orthogonal to the alkyne–azide "click" reaction of EdU-modified DNA. Alkene–tetrazine ligation reactions provide the first alternative to azide–alkyne click reactions for the bioorthogonal chemical labeling of nucleic acids in cells and facilitate time-resolved, multicolor labeling of DNA synthesis.*

Metabolic labeling and fluorescent detection of DNA are essential techniques for deciphering the timing and location of DNA synthesis in vivo.[1] The most commonly used label for this purpose is 5-bromo-2′-deoxyuridine (BrdU, **1**; Scheme 1 a). BrdU is incorporated into the DNA of dividing cells by endogenous enzymes and it can be detected with fluorescent antibodies upon permeabilization of the cells and denaturation of the DNA. However, the detection of BrdU is limited by the poor tissue permeability of antibodies.[2] Bioorthogonal chemical reactions provide attractive alternatives to immunohistochemical staining.[3] In this approach, a highly chemoselective reaction is used to ligate an exogenous probe to a metabolically labeled biomolecule.[4] For example, 5-ethynyl-2′-deoxyuridine (EdU, **2**; Scheme 1 a) can be detected by using a Cu^{I}-mediated azide–alkyne "click" cycloaddition (CuAAC) upon the addition of a fluorescent azide.[5] Since small-molecule fluorescent probes are utilized, EdU detection is more sensitive than the immunofluorescent detection of BrdU, especially in deep tissues. EdU itself, however, is highly toxic and its addition to living cells causes DNA damage, cell cycle arrest, and apoptosis.[6] To minimize these effects, "clickable" nucleosides that exhibit reduced toxicity, including F-*ara*-EdU (**3**; Scheme 1 a), have been developed.[2,7] All such bioorthogonal chemical reporters of DNA and RNA synthesis have hitherto utilized azide–alkyne click reactions. The development of alternative bioorthogonal chemical reactions for DNA imaging would be highly desirable in multidimensional pulse-chase labeling of DNA synthesis,[1,2] in experiments where CuAAC reactions are utilized for tagging other cellular components,[3,4] and for potentially minimizing the biological impact of the nucleoside analogue itself.[6]



Scheme 1. a) Thymidine analogues used for the metabolic labeling of DNA and their respective detection methods (underlined). b) The reaction between VdU (**4**) and 3,6-di-2-pyridyl-1,2,4,5-tetrazine **5** (py_2-Tz) in the presence of ambient oxygen. See the Supporting Information for the synthesis and characterization of compounds **4**, **6** and **7**.

Inverse electron demand Diels–Alder (invDA) reactions between electron-deficient tetrazines and electron-rich dienophiles are particularly attractive for bioorthogonal chemical reporter strategies since invDA reactions are irreversible, do not require a catalyst, and are compatible with cell media.[8] To date, invDA reactions have been used for labeling synthetic oligonucleotides in vitro, as well as cellular and cell-surface proteins, by using strained dienophiles such as norbornene,[8b–d,9] *trans*-cyclooctene,[9a,10] and cyclopropene.[11] The addition of such large substituents to nucleosides is known to inhibit their cellular metabolism.[12] We therefore sought the smallest possible dienophile as a bioorthogonal chemical reporter of cellular DNA synthesis. Since vinyl aromatic compounds are known to react with tetrazines,[13] we identified 5-vinyl-2′-deoxyuridine (VdU, **4**; Scheme 1 a) as a potential metabolic label for DNA.

To investigate the chemical reactivity of VdU in a model invDA reaction, VdU (**4**) and tetrazine **5** (1.2 eq) were incubated in a 2:1 mixture of 1,4-dioxane/water for 16 h at room temperature (Scheme 1 b). The reaction afforded dihydropyridazine **6** (VdU-Tz) as a mixture of tautomeric and diastereomeric isomers, as well as pyridazine **7** (VdU-Tz-ox), with an 81 % overall yield of isolated products (Scheme 1 b, and Scheme S3 and Figure S1 in the Supporting Information). Similar product mixtures have been reported for the strained alkenes that are widely used in cell-based conjugation reactions.[8–11] It was unclear, however, whether a terminal alkene such as VdU could exhibit sufficient reactivity towards tetrazines for the effective intracellular labeling of DNA.[14,15]

To evaluate the reaction kinetics of the VdU–tetrazine ligation as compared to styrene and 4-penteneamide, reactions were conducted under pseudo-first-order conditions by monitoring the consumption of tetrazine **5** upon the addition of each dienophile (Figure 1 and Figure S2 in the Supporting Information). 4-Penteneamide exhibited a relatively low rate constant (k) of $0.42 \times 10^{-2}\,M^{-1}s^{-1}$, while VdU and styrene exhibited 5- to 10-fold higher rates of $2.1 \times 10^{-2}\,M^{-1}s^{-1}$ and

[*] Dr. U. Rieder, Prof. Dr. N. W. Luedtke
Department of Chemistry, University of Zurich
Winterthurerstrasse 190, 8057 Zurich (Switzerland)
E-mail: nathan.luedtke@chem.uzh.ch
Homepage: http://www.bioorganic-chemistry.com

[**] We gratefully acknowledge the Swiss National Science Foundation (146754 to N.W.L.) and the University of Zürich Forschungskredit (FK-13-113 to U.R.) for financial support. We thank Dr. Kathrin Lang and Prof. Dr. Jason Chin for providing the fluorescent tetrazine "Tamra-Tz". We thank Dr. Anne B. Neef, Dr. Kai J. Neelsen and Therese Triemer for helpful discussions.

Supporting information for this article, including experimental details, is available on the WWW under http://dx.doi.org/10.1002/anie.201403580.

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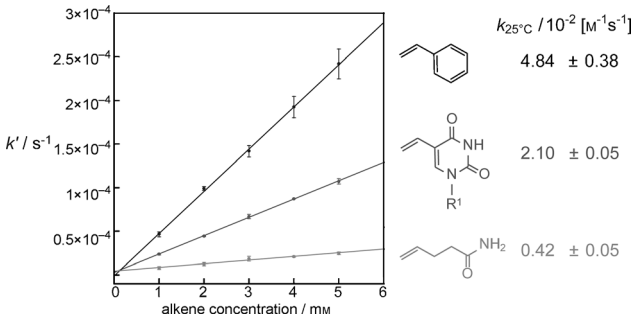

Figure 1. Pseudo-first-order reaction rates (k') versus alkene concentration for the consumption of tetrazine **5** (0.1 mM) in a 1:1 (v/v) mixture of methanol and water at 25 °C. The slope of each linear regression provides the rate constant k. R^1 = 2′-deoxyribose.

$4.8 \times 10^{-2}\,M^{-1}s^{-1}$, respectively (Figure 1). These values are lower than those reported for angle-strained olefins,[16] but are directly comparable to those of strain-promoted azide–alkyne cycloaddition "SPAAC" reactions ($k \approx 10^{-3}$–$10^{-1}\,M^{-1}s^{-1}$) widely used for cellular labeling purposes.[17]

To evaluate the ability of endogenous enzymes to incorporate VdU into DNA, HeLa cells were treated with variable doses of VdU for 16 h before being fixed and stained with a tetramethylrhodamine–tetrazine conjugate (Tamra-Tz; Scheme S1 in the Supporting Information).[8c] Dose-dependent nuclear staining was observed in cells treated with 1 to 100 μM of VdU (Figure 2 and Figure S3). Similar results were

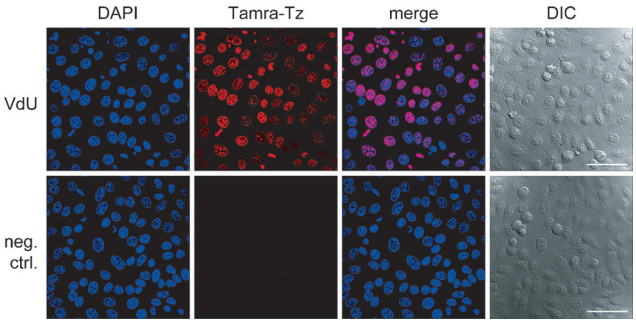

Figure 2. Bioorthogonal invDA ligation of VdU in newly synthesized DNA upon the addition of the fluorescent tetrazine Tamra-Tz. HeLa cells were treated with 30 μM VdU for 16 h, followed by fixation, DNA denaturation, and incubation with 5 μM Tamra-Tz for 4 h. Total cellular DNA was stained with DAPI. The negative control (neg. ctrl.) samples received identical treatment but were not incubated with VdU prior to staining. Scale bars: 50 μm. DIC = differential interference contrast image, merge = overlay of Tamra-Tz and DAPI channels.

observed in U2OS, A549, Vero, and MRC-5 cells (Figures S4–S7). The addition of 5 μM Tamra-Tz to VdU-treated cells resulted in rapid intranuclear staining after only 30 min at 37 °C (Figure S8). To evaluate the selectivity of VdU for incorporation into cellular DNA versus RNA, living HeLa cells were treated with 30 μM of VdU in the presence of aphidicolin, an inhibitor of DNA synthesis, for 16 h. Subsequent staining revealed no detectable VdU labeling (Figure S9). Together, these results demonstrate selective metabolic incorporation of VdU into the DNA of replicating cells.

Metabolic labeling experiments can last anywhere from minutes to weeks prior to fixation and analysis.[1,2] The biological impact of the metabolic label should thus be minimal during this labeling period. In the case of EdU, experimental results can be perturbed by DNA damage and cell cycle arrest.[2,6] To assess the cytotoxicity of VdU as compared to EdU, cell cultures were grown in the presence of various nucleoside concentrations for 24–72 h, and total metabolic activities were measured by using the Alamar Blue assay. In all of the cell lines tested, EdU was 2- to 15-fold more toxic than VdU (Figure S10 and Table S1 in the Supporting Information). When HeLa cells were incubated with the standard labeling concentration of EdU (10 μM) for 4–16 h, no changes in cellular morphology or total cellular respiration were observed, but a dramatic accumulation of tetraploid (4n) cells that stained positively for the phosphorylation of histone H2AX was detected (Figure 3). This

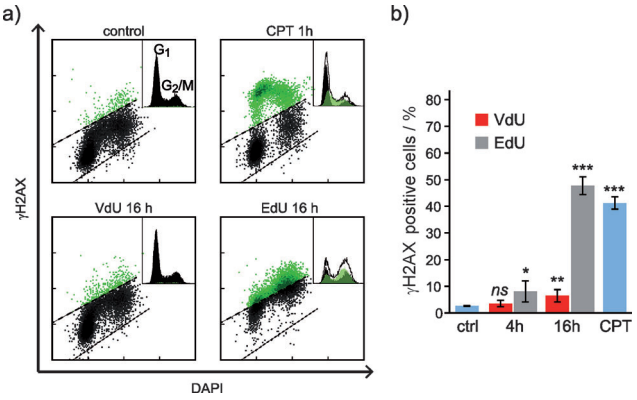

Figure 3. Flow cytometry analysis of γH2AX versus DAPI staining of HeLa cells treated with 30 μM VdU or 10 μM EdU for 16 h. These concentrations were selected based upon the equivalent staining frequencies and intensities observed for EdU and VdU (Figure 4b,c, and Figure S15,16). γH2AX positive cells were detected by using a phosphospecific antibody. a) The dot plots illustrate γH2AX abundance versus the total cellular DNA content of each cell. The insets display histograms of the DNA content (from the cell cycle phases $G_0/_1$ to G_2/M) versus cell count. The dashed lines show the thresholds used for defining γH2AX positive/negative cells based on the control. For a positive control, cells were treated for 1 h with camptothecin (CPT, 0.5 μM), which causes DNA damage.[21] b) A graphical representation of results where $n = 5$, $^*P < 0.02$, $^{**}P < 0.002$, $^{***}P < 0.0001$, ns = not significant, compared to the control. For 4 h time points and U2OS cells see Figure S11.

γH2AX formation is associated with DNA strand breakage.[18] Similar results were obtained with U2OS cells (Figure S11). By contrast, little or no γH2AX formation or G_2/M cell cycle arrest was observed in cells treated with 30 μM of VdU (Figure 3 and Figure S11). While the reasons for these differences are unclear, previous studies have established a relationship between the chemical stability of modified nucleotides and their ability to initiate the DNA damage response.[19] EdU is known to initiate spontaneous DNA strand breaks in the presence of nucleophilic amines.[20] Interestingly, EdU (**2**) decomposed with a half-life of 8 h in a 20% aqueous methyl amine solution at room temperature, while VdU (**4**) exhibited no detectable decomposition even after 48 h (Figure S12).

Pulse-chase labeling experiments, in which multiple metabolic probes are introduced into DNA over time, are used in a wide variety of important biological investigations,

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such as characterizing the timing of DNA replication, visualizing embryogenesis, and in stem cell research.[1,2] We therefore examined the possibility of using VdU–tetrazine ligation in combination with EdU–azide cycloaddition for introducing orthogonal chemical labels into cellular DNA. One potential pitfall to this approach is that alkynes can cross-react with tetrazines.[13,16] The reactivity of tetrazine **5** towards EdU was therefore evaluated in vitro (Figure 4a). The addition of excess VdU (**4**) caused rapid consumption of tetrazine **5**, but no reaction was observed with EdU (**2**). This finding was confirmed by NMR experiments conducted over 48 h (Figure S13). These results demonstrate the feasibility of orthogonal DNA labeling with both VdU and EdU.

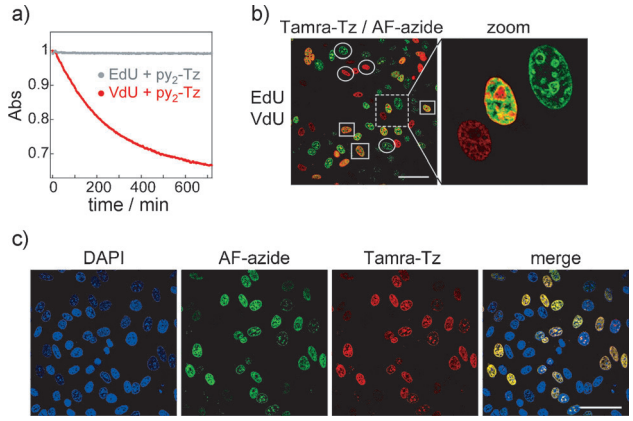

Figure 4. a) The consumption of tetrazine **5** (0.1 mM) according to normalized absorbance changes at 530 nm in the presence of 5 mM of EdU or VdU in a 1:1 (*v*/*v*) mixture of methanol and water at 25 °C. b) Confocal fluorescence microscopy images of cells treated sequentially with 10 μM EdU (4 h) and 30 μM VdU (4 h), followed by fixation, DNA denaturation, and staining with Tamra-Tz (red) and AF-azide (green). The overlay of the two color channels reveals both distinct and dual labeling, indicated by circles and squares, respectively. c) Cells were treated simultaneously with a mixture of VdU (30 μM) and EdU (10 μM) for 8 h and stained as in (b). Scale bars: 50 μm; merge = overlay of AF-azide, Tamra-Tz, and DAPI channels. See Figures S15,16 for additional control experiments and quantitative image analyses.

To further evaluate the relative efficiencies of nucleoside incorporation and detection, optimized concentrations of VdU (30 μM) and EdU (10 μM) were prepared as a mixture and co-administered to cells for 8 h. Following staining, extensive colocalization of VdU (red) and EdU (green) with approximately equal staining intensities were observed, thereby giving a yellow color in the merged images (Figure 4c). Taken together, these results indicate that the methods used for the chemical detection of VdU and EdU are both efficient and mutually orthogonal in cellular DNA.

The orthogonal chemical labeling of VdU and EdU could, in principal, be expanded to a third level of spatial and temporal resolution if the immunohistochemical staining of BrdU was included. Previous studies, however, have demonstrated that BrdU and EdU are often incompatible because EdU pulses can inhibit the subsequent incorporation of BrdU[2] and most anti-BrdU antibodies (≈90%) exhibit cross-reactivity with EdU.[22] We therefore selected F-*ara*-EdU as a relatively nontoxic EdU analogue (Figure S11C,D) that is compatible with subsequent BrdU incorporation and detection.[2] To investigate the compatibility of VdU with BrdU and F-*ara*-EdU, HeLa cells were sequentially treated with BrdU (30 μM), VdU (30 μM), and F-*ara*-EdU (10 μM). Subsequent staining revealed well-resolved, non-overlapping fluorescent signals (Figure 5 and Figure S17). The spatial and

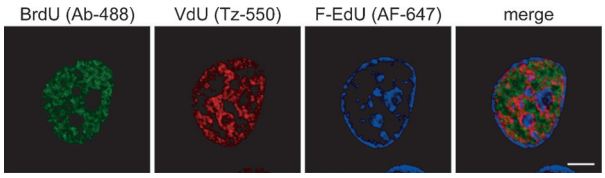

Figure 5. Visualizing the progression of the S-phase in a single cell by using BrdU (30 μM, 1st pulse, green), VdU (30 μM, 2nd pulse, red), and F-*ara*-EdU (10 μM, 3rd pulse, blue). HeLa cells were incubated for 2 h 45 min with each nucleoside and washed for 15 min with fresh media in between nucleoside treatments. After fixation and DNA denaturation, the cells were stained with Tamra-Tz (red), BrdU antibody (green), and AF-azide (blue). Scale bars: 5 μm. See Figure S17 for additional examples.

To test the chemical orthogonality of VdU and EdU in the context of cellular DNA, HeLa cells were independently treated with EdU or VdU before being fixed and stained with Tamra-Tz followed by azide-modified AlexaFluor 488 (AF-azide) under CuAAC conditions. The ligation reactions did not show any cross-reactivity since Tamra-Tz did not label EdU-treated cells and VdU-treated cells were not stained by AF-azide (Figure S14). To evaluate the orthogonality of VdU and EdU in individual cells containing one or both labels, cells were treated sequentially with a pulse of EdU for 4 h followed by a VdU "chase" for an additional 4 h. The cells were then fixed and stained with Tamra-Tz and AF-azide. The resulting staining revealed cells containing only one of the two labels (circles, Figure 4b), as well as cells with well-resolved signals from both labels (squares, Figure 4b). Quantitative image analyses indicated that VdU and EdU were incorporated and detected in the cells with similar efficiencies (Figures S15,16). temporal distribution of the labels were characteristic of S-phase progression,[23] where the first label stained euchromatin in the early S-phase as numerous small replication foci throughout the nucleus, the second label was observed at a fewer number of larger foci located around the nucleoli, and the final label gave staining in distinct globular regions at the nuclear envelope and perinucleolar heterochromatin corresponding to the late S-phase. The order of nucleoside addition did not influence these patterns, thereby demonstrating that none of the three nucleosides interfered with metabolism or the detection of the other two (Figure S17).

In conclusion, VdU is selectively incorporated into cellular DNA, where it can be detected by using alkene–tetrazine ligation. This is the first example of an inverse electron demand Diels–Alder reaction on cellular nucleic acids. The reaction rate between VdU (**4**) and dipyridyl tetrazine **5** is similar to those for strain-promoted azide–

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alkyne cycloaddition "SPAAC" reactions,[17d–f] and the addition of Tamra-Tz to VdU-treated cells results in rapid intranuclear staining apparent after only 30 min. These results demonstrate that highly reactive strained alkenes are not a prerequisite for effective intracellular labeling through tetrazine ligation.[9–11,14] The biological properties of VdU are highly desirable since it causes relatively little γH2AX formation or cell cycle arrest when it is applied at optimal labeling concentrations. These results open the door to alternative bioorthogonal chemical reactions for the modification of cellular nucleic acids, for example, tetrazole photoligation[24] or palladium-catalyzed Heck reactions.[25]

Received: March 21, 2014
Published online: June 30, 2014

Keywords: bioorthogonal reporters · Diels–Alder cycloaddition · fluorescent probes · metabolic labeling · nucleosides

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