FINAL REPORT TO MULTIPLE LABELING OF DNA AND RNA WITH NIR FLUOROPHORES BY MEANS OF (BIO)ORTHOGONAL APPROACHES

Within this project we aimed at developing biorthogonal reagents and bioorthogonalized fluorescent probes for cellular imaging of DNA. Such optically functionalized DNA architectures can be used for the better understanding of biomolecular processes or in diagnostic processes. We proposed the development of a generally applicable technique for the implementation of nucleotides into short and longer sequences (DNA or RNA) that enable post-synthetic incorporation of fluorescent / fluorogenic tags. Upon systematic examination and development of biorthogonal reagents we have synthesized phosphoramidite derivatives furnished with appropriate bioorthogonal functions that enabled incorporation of such building blocks into short oligos by automated DNA synthesis. Furthermore, we also investigated the possibility of incorporation of as-functionalized nucleotide triphosphates into longer oligos by means of DNA polymerases. Parallel to this work, we have accomplished the synthesis and study of a set of near-IR emitting or fluorogenic labels that offer post-synthetic optical manipulation od DNA (RNA) by means of biorthogonal transformations.

In the proposal we aimed to accomplish the following tasks:

- Development of activated tetrazines for fast reactions with simple dienophiles
- Development of new, highly reactive cyclooctyne reagents for metal-free azide-alkyne cycloaddition
- Design and synthesis of bioorthogonally applicable fluorogenic (*turn-on*), NIR, mega-Stokes tags
- Synthesis of simple dienophile-modified, reactive tetrazine modified nucleoside phosphoramidites for automated DNA/RNA synthesis and nucleoside triphosphates for polymerase-assisted (and even metabolic) incorporation of bioorthogonal functions
- Bioorthogonal labeling of modified oligonucleotides with fluorogenic / NIR fluorescent tags to explore labeling DNA for live cell imaging especially delivery to cells

RESULTS

1. *Development of activated tetrazines for fast reactions with simple dienophiles*

In order to search for reactive yet simple tetrazine scaffolds suitable for DNA-based conjugation studies, we have initiated a study that examined the reactivity and stability of such dienes as a function of their electronic and steric features. To this end a set of nicotinic acid derivatized tetrazines were synthesized and evaluated for activity in inverse electron demand Diels-Alder (IEDDA) reactions with various dienophiles (cyclooctnyes, enol, *trans*-cyclooctene) (Figure 1). It was found that the performance of these tetrazines is governed by both factors, however, theoretical and experimental investigations showed that steric effects may override the energetically predicted order of reactivity.[1]

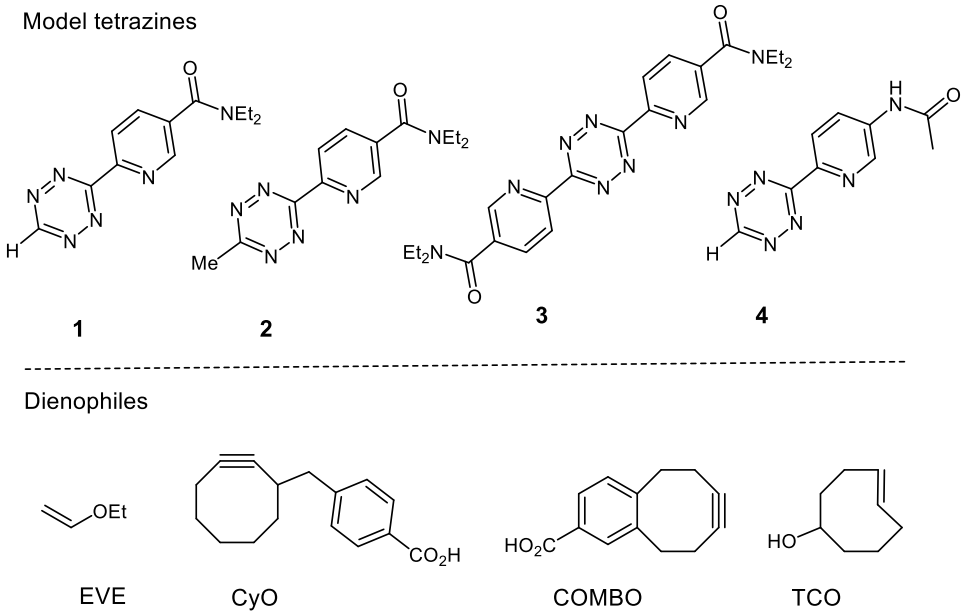
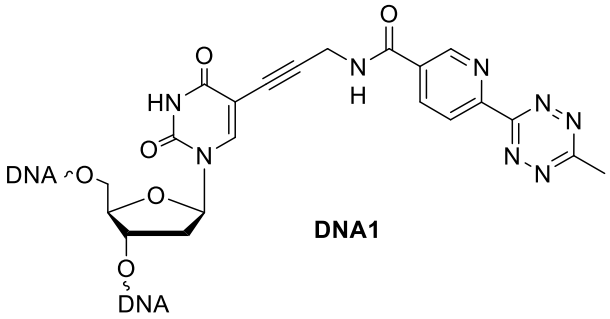


Figure 1. Structure of model tetrazines with different steric demand (top) and dienophiles used in this study (bottom).

Making a compromise between reactivity and stability a selected tetrazine scaffold (**2**) was incorporated into a deoxynucleotide to afford a bioorthogonalized bulding block enabling iEDDA based tagging schemes of nucleic acids. Incorporation of this modified building block into a DNA sequence using standard solid phase nucleic acid synthesis, a fully automated protocol together with subsequent on-bead labeling with a fluorophore (fluorescein, Flu) bearing a reactive dienophile (a cyclooctyne, termed as COMBO) revealed that this tetrazine is suitable for the implementation of a reactive bioorthogonal handle into DNA (Figure 2).

[1] G. Cserép, O. Demeter, E. Bätzner, M. Kállay, H.-A. Wagenknecht, P. Kele Synthesis and Evaluation of Nicotinic Acid Derived Tetrazines for Bioorthogonal Labeling. *Synthesis* **2015**, *47*, 2738-2744.



DNA1 3'−A-G-T-C-A-C-T-T−**8**−T-T-C-T-G-A-C-G−5'
DNA1c 5'−T−C-A-G-T-G-A-A−A-A-A-G-A-C-T-G-C−3'

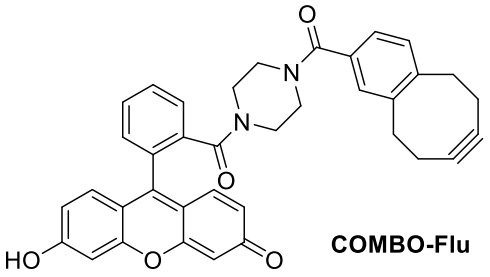


Figure 2. Sequence of DNA1 with the tetrazine building block **8** that reacts rapidly with the cycloocytyne-modified fluoresceine (COMBO-Flu).

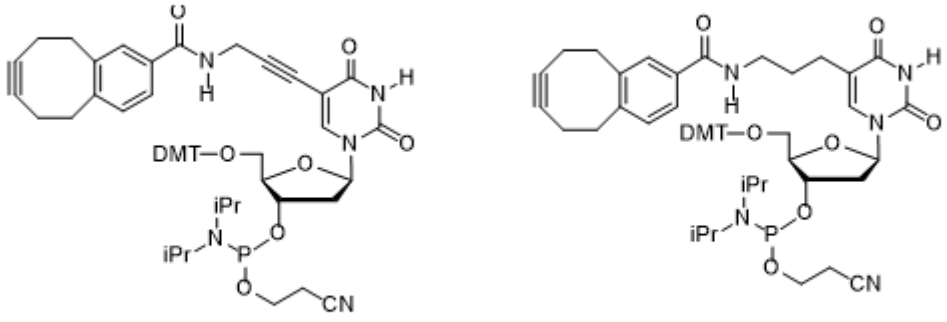
2. Development of new, highly reactive cyclooctyne reagents for metal-free azide-alkyne cycloaddition

We have developed a reactive, relatively hydrophilic cyclooctyne derivative (COMBO) suitable for either azide-alkyne or tetrazine-cyclooctyne type of ligation schemes. COMBO was successfully applied in various studies including the one in the preceeding paragraph. Besides, Rhodamine-COMBO was applied in the tagging scheme of genetically manipulated amphiphilic proteins capable of self-assembly to artificial vesicles.[2] Furthermore, COMBO was attached to a 2'-deoxyuridine and incorporated into DNA by standard phosphoramidite chemistry. The reactive group allowed rapid, efficient and copper-free postsynthetic modification as demonstrated in a reaction with a fluorescent quinolinium-styryl-coumarin azide dye also developed in our laboratory.[3] The COMBO group attached to the 5-position of a 2'-deoxyuridine in phosphoramidite **5** allows rapid and efficient copper-free postsynthetic modification of DNA as demonstrated with a far-red emitting fluorescent azide probe. Upon labeling strong fluorescence intensity enhancement is observed. However, the fluorescence quantum yield of

[2] M. C. Huber, A. Schreiber, P. von Olshausen, B. R. Varga, O. Kretz, B. Joch, S. Barnert, R. Schubert, S. Eimer, P. Kele, S. M. Schiller Designer amphiphilic proteins as building blocks for the intracellular formation of organelle-like compartments. *Nat. Mater*. **2015**, *14*, 125-132.

[3] C. Stubinitzky, G. B. Cserép, E. Bätzner, P. Kele, H-A. Wagenknecht 2'-Deoxyuridine Conjugated with a Reactive Monobenzocyclooctyne as a DNA Building Block for Copper-Free Click-type Postsynthetic Modification of DNA. *Chem. Commun*. **2014**, *50*, 11218-11221.

such modified DNA double strands was unexpectedly low, which was attributed to the rather rigid and inflexible ethynyl linker between the 2'-deoxyuridine part and the fluorophore which interferes with best possible interactions of the dye with the DNA. Hence, a new phosphoramidite **6** that also carries the COMBO group attached to the 5-position of 2'-deoxyuridine but linked via a saturated alkyl and thereby a flexible linker was synthesized, and extensively applied for NIR labeling (Figure 4).



5 **6**

Figure 3. Phosphoramidites **5** and **6** bearing COMBO motif.

At the final stage of the project we have changed the target dienophile scaffolds to *trans*-cyclooctene (TCO) motifs as these enable considerably faster reactions with tetrazines in inverse electron demand Diels-Alder reactions compared to azide-alkyne transformations. Thus, we have started the development of hydrophilic TCO motifs.

3. *Design and synthesis of bioorthogonally applicable fluorogenic (turn-on), NIR, mega-Stokes tags*

We focused on two kinds of fluorogenic scaffolds: 1) Aromatic azides where the azide plays a dual role: acts as a biorthogonal handle and a quencher of fluorescence. The quenching ability of the azide function was investigated theoretically and experimentally. These studies led to the synthesis of fluorogenic azides that considerably reduced background fluorescence. These labels were successfully employed in the labeling schemes of genetically manipulated (e.g. cyclooctynylated) proteins.[4] We have also developed polarity sensitive aromatic azides whose fluorescence increased only upon environmental changes e.g. binding to DNA sequences.[5] Two series of new, water soluble, membrane-permeable, far-red / NIR emitting benzothiazolium-based fluorescent labels with large Stokes-shifts

[4] A. Herner, G. E. Girona, I. Nikić, M. Kállay, E. A. Lemke, P. Kele New Generation of Bioorthogonally Applicable Fluorogenic Dyes with Visible Excitations and Large Stokes Shifts. *Bioconjugate Chem*. **2014**, *25*, 1370-1374.

[5] Á. Eördögh, J. Steinmeyer, K. Peewasan, U. Schepers, H-A. Wagenknecht, P. Kele Polarity sensitive bioorthogonally applicable far-red emitting labels for postsynthetic nucleic acid labeling by copper-catalyzed and copper-free cycloaddition. *Bioconjugate Chem*. **2016**, *27*, 457-464.

were conjugated to alkyne-modified oligonucleotides through their azide moiety via CuAAC. Of the dyes tested one set showed remarkable fluorescence intensity enhancement upon conjugation to DNAs. We also incorporated COMBO motif containing phosphoramidite **6** into the DNAs, thus enable copper-free labeling schemes. We have also tested the *in vivo* labeling potential of these newly synthesized dyes on HeLa cells previously transfected with cyclooctynylated DNA. Confocal fluorescent images showed that the dyes are all able to cross the membrane and suitable for background-fluorescence free fluorescent tagging of nucleic acids. Moreover, we have observed different accumulation of the two dye series in the endosomal particles, or in the nuclei, respectively (Figure 4).

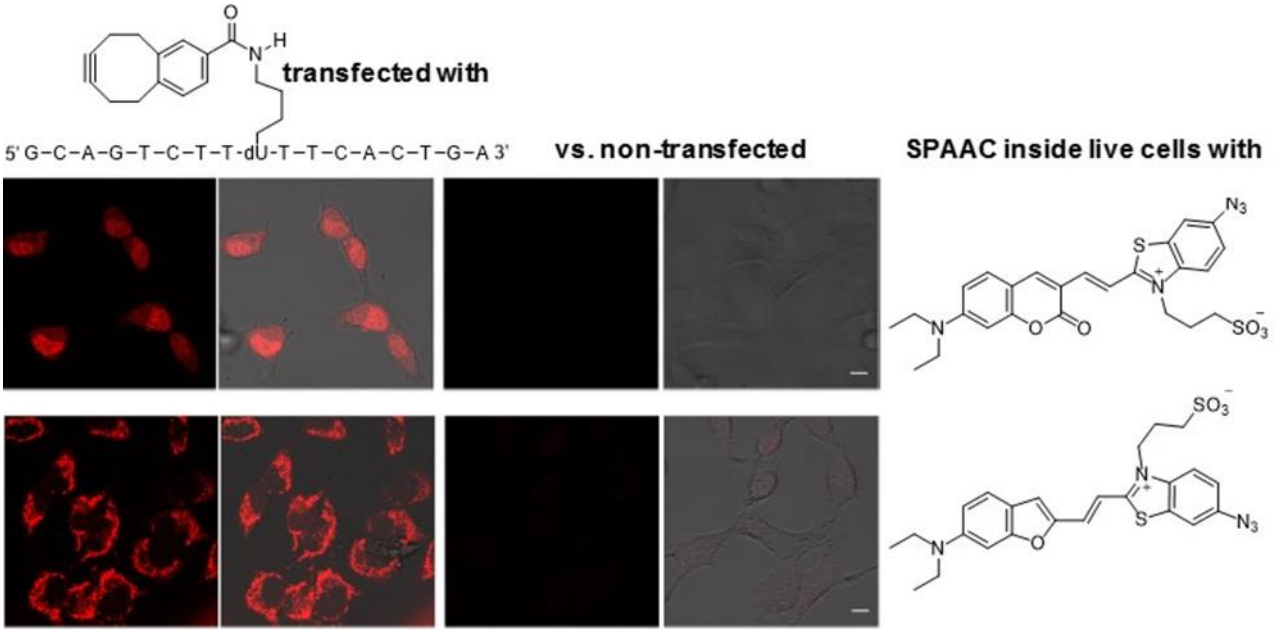


Figure 4. Representative images of HeLa cells transfected with COMBO-modified DNA. Only transfected cells undergo strain-promoted azide-alkyne cycloaddition with azido dyes.

At later stage of the project we also developed bisazide, thus bis-quenched fluorogenic scaffolds (**7**). These scaffolds enabled more specific two-point binding to bis-cyclooctynylated motifs and featured much higher fluorogenicity (i.e. 200 fold fluorescence increase upon binding) (Figure 5).[6]

[6] O. Demeter, E. A. Fodor, M. Kállay, G. Mező, K. Németh, P. T. Szabó, P. Kele A Double-Clicking Bis-Azide Fluorogenic Dye for Bioorthogonal Self-Labeling Peptide Tags. *Chem. Eur. J*. **2016**, *22*, 6382-6388.

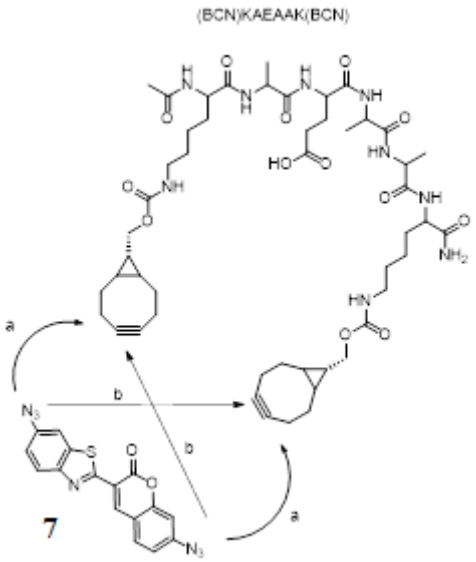


Figure 5. Representative example for the formation of cyclic peptide-**7** conjugates. Possible reactions between probe **7** and target peptide (BCN)KAEAAK(BCN). Note that in either case a cyclic bis-clicked product is formed.

In line with our topic change to *trans*-cycloctene dienophiles we have started the development of fluorogenic scaffolds bearing tetrazine motif. In our recent study it was demonstrated that near-infra red emitting phenoxazine scaffolds linked to tetrazines via a phenylene or vinylene linker are extremely efficient fluorogenic probes. The quenching in this case is the most efficient mode to diminish fluorescence as it works via through-bond-energy-transfer (TBET) mechanism. The developed phenoxazines turned out to be membrane permeable, NIR emitting and offered background free fluorescence imaging of genetically altered (e.g. *trans*-cyclooctenylated) proteins (Figure 6).[7]

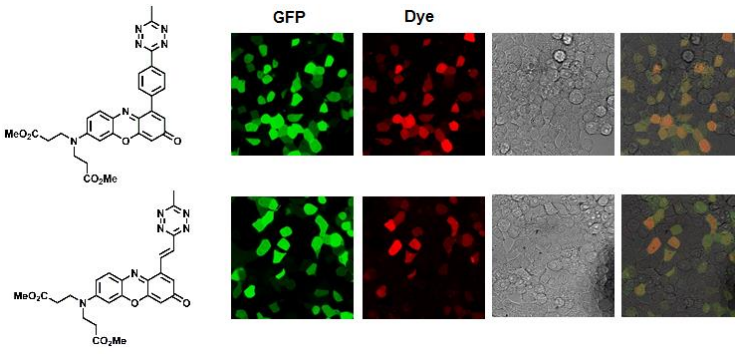


Figure 6. Confocal microscopy images of GFP(Y39TAG→TCO*) expressing HEK293T cells treated live with TBET-fluorogenic phenoxazine dyes for 1h.

[7] G. Knorr, E. Kozma, A. Herner, E. A. Lemke, P. Kele New, red-emitting tetrazine-phenoxazine fluorogenic labels for live-cell intracellular bioorthogonal labeling schemes. *Chem. Eur. J*. **2016**, 22, 8972-8979.

4. Nucleoside triphosphates for polymerase-assisted incorporation of bioorthogonal functions

In order to extend the biorthogonal toolbox for polymerase assisted nucleic acid synthesis, we synthesized the nucleoside triphosphates **8-11** and the 2'-deoxyuridine triphosphates **12-15** which carry different bioorthogonally reactive groups (Figure 7). Incorporation of these modified building blocks was performed as primer extension experiments to test the applicability and tolerance of different DNA polymerases together with the postsynthetic labeling potential with correspondingly functionalized fluorophores. The typical setup for the primer extension experiments consisted of the primer with 23 nucleotides length and a fluorescein label at the 5'-end to facilitate detection by gel electrophoresis. Two templates with 35 nucleotides length were designed for standing start and running start experiments, respectively. Both templates bear only one adenosine, either at position 24 (standing start) or at position 27 (running start) to selectively insert **8** or **12-15** at distinct positions. All applied polymerases lacked the 3'-5'-exonuclease activity. These experiments revealed the scope and limitations of important bioorthogonal reactions with respect to nucleic acid modification. **8**, **9**, **10** and **11** were successfully incorporated in oligonucleotides using 9°N and Therminator DNA polymerases. Most importantly, the ethynyl group as single 2'-modification of the enzymatically prepared oligonucleotides can be applied for postsynthetic labeling. This was representatively shown by PAGE analysis after Cu(I) catalyzed click reaction with a fluorescent nile-red azide. These results are important since they show that the 2'-position as one of the most important modification site in oligonucleotides is now accessible not only for synthetic but also for enzymatic oligonucleotide preparation. The other functionalities of **12-15** were tolerated, incorporated and, most importantly, bypassed by standard DNA polymerases, such as Vent(exo), Deep Vent(exo) and Hemo KlenTaq. The "photoclick" reaction of an oligonucleotide prepared with tetrazole **12** was labeled with a maleimide-modified dye in quantitative yield. For the Diels-Alder-type modification, oligonucleotides that were prepared with cyclopropene **13** were successfully labeled with tetrazine-dyes in 60-70% yields. On the other hand, oligonucleotides that were prepared with tetrazines **14** and **15** and were treated with COMBO and bicyclononyne-modified rhodamine dyes, worked but only in low yields. Several attempts to improve the yields failed. It is likely that the stability of the tetrazine moiety is not sufficient to quantitatively "survive" the conditions of primer extension. These latter results clearly show the limitation of bioorthogonal reactions with respect to nucleic acid modification. Especially tetrazines fail not only in phosphoramidites as chemical DNA building blocks (except the solid phase protocol published in ref. 1), but also as triphosphates for biochemical DNA preparation. Of the triphosphates mentioned in this section, **13** and **15** were prepared by the Hungarian group along with all the fluorescent dyes applied.[8]

[8] M. Merkel, S. Arndt, K. Peewasan, G. B. Cserép, Ulrike Wenge, P. Kele, H.-A. Wagenknecht Scope and limitations of typical biorthogonal reactions with nucleic acids: A status report on new 2'-deoxyuridine triphosphates for DNA primer extension and postsynthetic labeling. *J. Org. Chem*. **2016**, **accepted with revision**

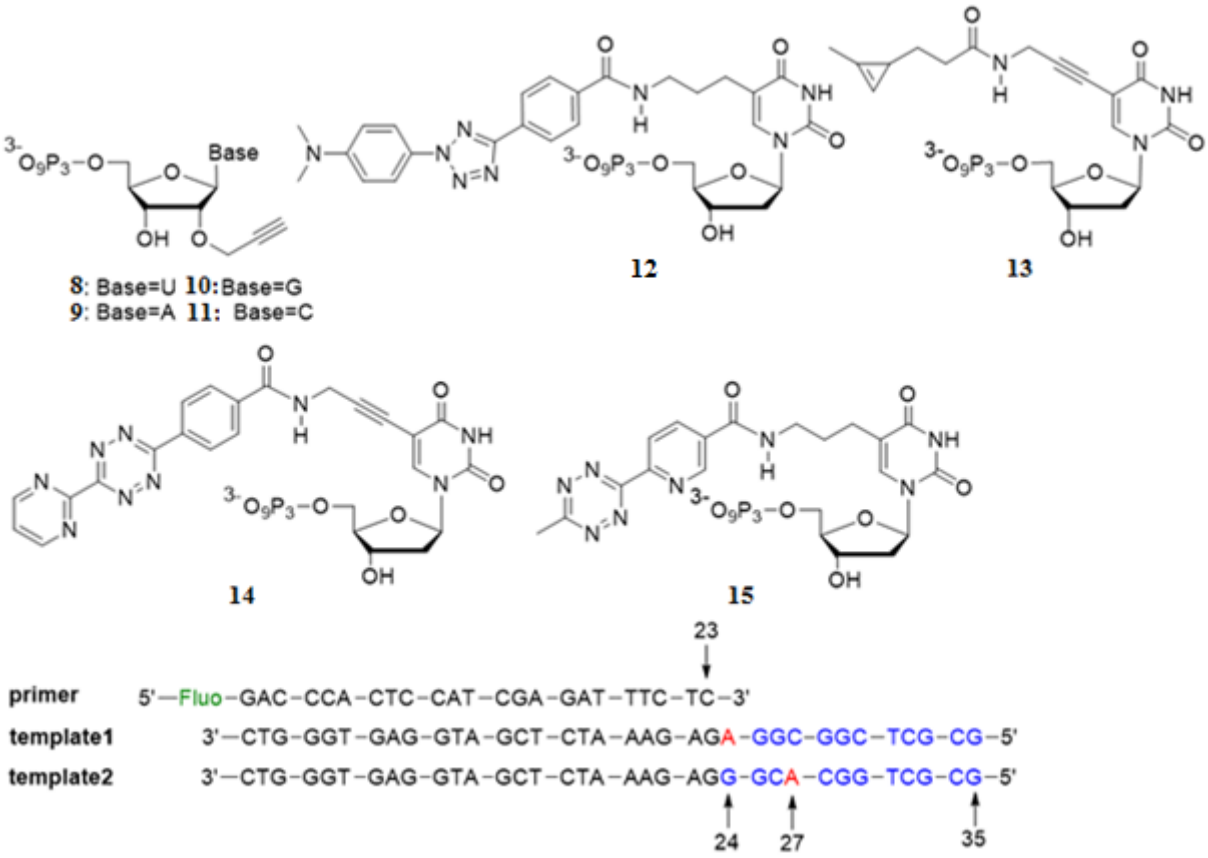


Figure 7. Synthesized triphosphate building blocks bearing biorthogonally reactive groups and typical setup for primer extension experiments.

5. Cys and Tyr selective biorthogonal tags

Part of this project, linkers (called chemical reporters) were also developed. These chemical reporters enabled specific targeting of rare amino acid side chains (cysteine or tyrosine) with specific warheads on the one hand, and carried a biorthogonal function on the other (cyclooctyne, azide, terminal alkyne). In various studies we have demonstrated the use of such linkers in two step, sequential protein labeling schemes using fluorogenic labels (Figure 7).[9, 10, 11]

[9] G. B.Cserép, A. Herner, O. S. Wolfbeis, P. Kele Tyrosine specific sequential labeling of proteins. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 5776-5778.
[10] G. B. Cserép, Zs. Baranyai, D. Komáromy, K. Horváti, Sz. Bősze, P. Kele Fluorogenic tagging of peptides via Cys residues using thiol-specific vinyl sulfone affinity tags. *Tetrahedron* **2014**, *70*, 5961-5965.
[11] B. Söveges, T. Imre, T. Szende, Á. L. Póti, G. B. Cserép, T. Hegedűs, P. Kele, K. Németh Systematic study of protein labeling by fluorogenic probes using cysteine targeting vinyl sulfone-cyclooctyne tags. Org. Biomol. Chem. **2016**, *14*, 6071-6078.

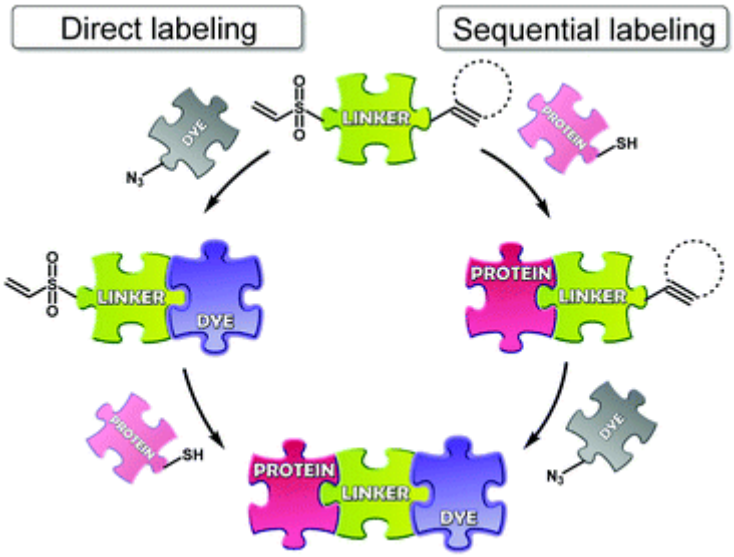


Figure 6. Comparison of direct and sequential fluorescent modification schemes.

Part of the project we have also contributed to a special issue on bioconjugation of probes in Methods and Applications in Fluorescence with a review paper.[12]

PRESENTATION OF RESULTS:

Besides the referred publications (11 publications, total IF: 79.892 and one more manuscript at J. Org. Chem. is accepted with revision, but still pending) we have presented several posters and oral presentations at different events (International: EMBL Chemical Biology, Heidelberg, 2014, ICBS & ECBS Berlin, 2015, Blue Danube Heterocyclic Symposium, Olomouc, 2013; National: Heterocyclic and Bioorganic Chemistry Workshop, Balatonszemes, 2013, 2014, 2015, 2016). Furthermore, the results were also accounted on invited lectures at various universities: Paris, Freiburg, Heidelberg, Karlsruhe, Pforzheim).

During the funding period one PhD student from each group spent a month at the collaborative partner's lab.

During the period of the project 3 PhD, 3 MSc, and 12 BSc degrees were obtained.

[12] G. B. Cserép, A. Herner, P. Kele Bioorthogonal fluorescent labels: a review on combined forces. *Methods Appl. Fluoresc.* **2015**, *3*, 042001.