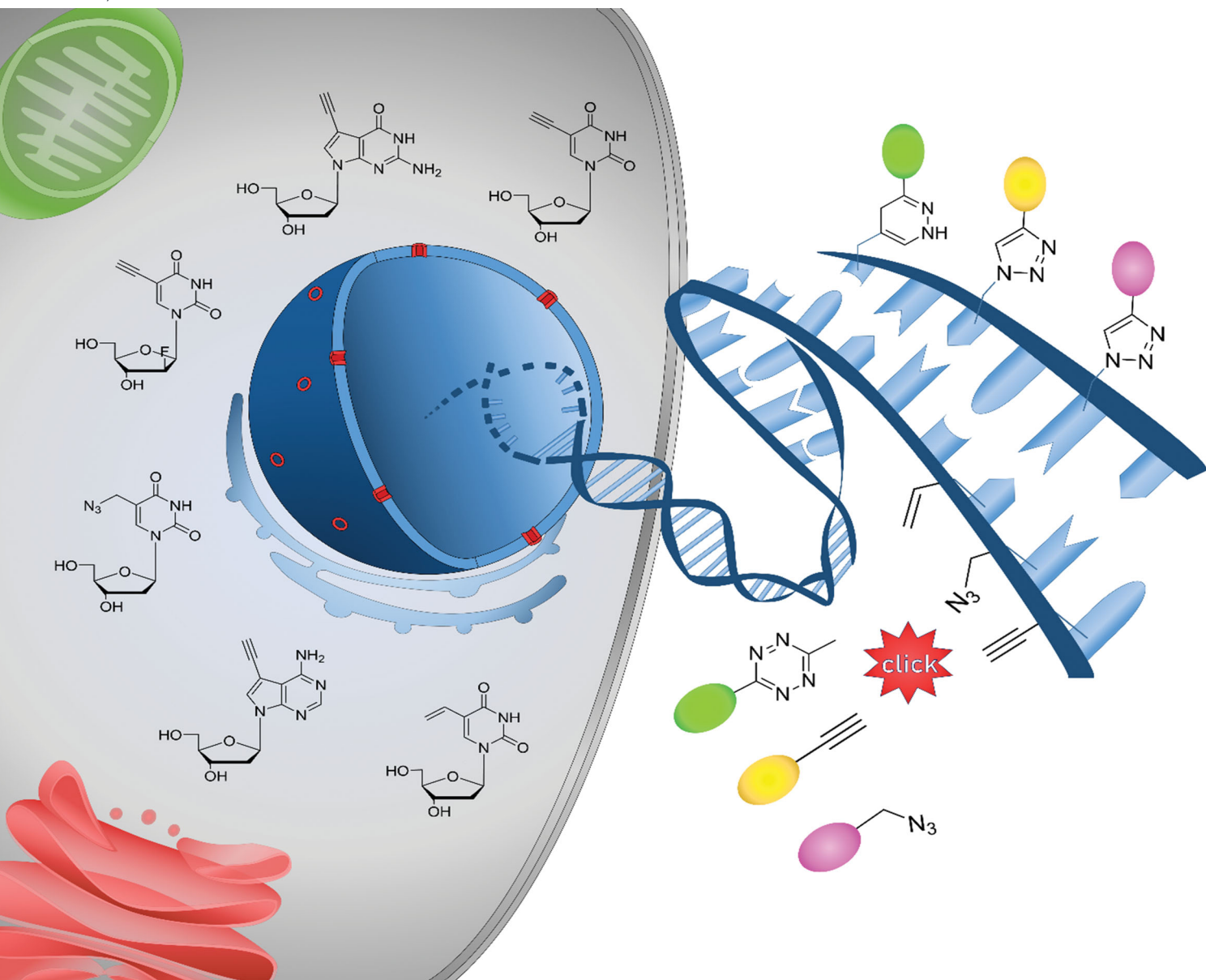


RSC Chemical Biology

rsc.li/rsc-chembio



ISSN 2633-0679

REVIEW ARTICLE

Hans-Achim Wagenknecht *et al.*
Labelling of DNA and RNA in the cellular environment by
means of bioorthogonal cycloaddition chemistry

REVIEW

[View Article Online](#)
[View Journal](#) | [View Issue](#)Cite this: *RSC Chem. Biol.*, 2020, 1, 86Received 16th April 2020,
Accepted 27th May 2020

DOI: 10.1039/d0cb00047g

rsc.li/rsc-chembio

Labelling of DNA and RNA in the cellular environment by means of bioorthogonal cycloaddition chemistry

Dorothee Ganz, Dennis Harijan and Hans-Achim Wagenknecht *

Labelling of nucleic acids as biologically important cellular components is a crucial prerequisite for the visualization and understanding of biological processes. Efficient bioorthogonal chemistry and in particular cycloadditions fulfill the requirements for cellular applications. The broadly applied Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), however, is limited to labellings *in vitro* and in fixed cells due to the cytotoxicity of copper salts. Currently, there are three types of copper-free cycloadditions used for nucleic acid labelling in the cellular environment: (i) the ring-strain promoted azide–alkyne cycloaddition (SPAAC), (ii) the “photoclick” 1,3-dipolar cycloadditions, and (iii) the Diels–Alder reactions with inverse electron demand (IEDDA). We review only those building blocks for chemical synthesis on solid phase of DNA and RNA and for enzymatic DNA and RNA preparation, which were applied for labelling of DNA and RNA *in situ* or *in vivo*, i.e. in the cellular environment, in fixed or in living cells, by the use of bioorthogonal cycloaddition chemistry. Additionally, we review the current status of orthogonal dual and triple labelling of DNA and RNA *in vitro* to demonstrate their potential for future applications *in situ* or *in vivo*.

Introduction

Labelling of cellular components is a crucial prerequisite for the visualization and understanding of biological processes.^{1,2} Among the different cellular components, proteins represent

Institute of Organic Chemistry, Karlsruhe Institute of Technology (KIT),
Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany. E-mail: Wagenknecht@kit.edu

**Dorothee Ganz**

of Organic Chemistry at KIT in 2019 and works under the supervision of Hans-Achim Wagenknecht. She follows her interests in chemical biology and works on metabolic labelling of nucleic acids by bioorthogonally reactive building blocks in order to investigate intracellular metabolic processes.

Dorothee Ganz was trained as a medical-technical laboratory assistant, she studied Chemical Biology at the Karlsruhe Institute of Technology (KIT, Germany) and obtained the MSc degree in the research area of organ-on-a-chip models. Through her years of work in the microbiology department of the Municipal Clinic Karlsruhe, she also gained experience in the field of medical microbiology. For her doctoral research she joined the Institute

**Dennis Harijan**

develops new cyclopropane- and cyclopropenone-modified nucleosides for the metabolic labelling of nucleic acids.

Dennis Harijan received his BSc degree in Chemical Biology at the Karlsruhe Institute of Technology (KIT, Germany) under the supervision of Anne S. Ulrich in 2016. Then he moved into the field of nucleic acid chemistry and investigated new building blocks for the bioorthogonal labelling of DNA and RNA. He obtained the MSc degree in 2018 and is currently working as a doctoral researcher under the supervision of Hans-Achim Wagenknecht and



the main carrier of biological functions. Hence, it is not surprising that the chemical toolbox for protein labelling is well developed.^{3–7} Among the non-proteinaceous biomolecules, nucleic acids play a central role for cellular functions and regulations. DNA is the carrier of genetic information and is regulated by epigenetic modifications.⁸ RNA is central for transcription and translation of the genetic information into the protein function. Moreover, the function of non-coding RNAs, such as miRNA,⁹ lncRNA,¹⁰ and eRNA,^{11,12} is currently an increasingly important topic of research, and their imaging and detection inside cells are challenges for future research. It is of utmost importance to label and image those biomolecules “beyond the proteome”,¹³ inside their natural cellular environment.^{5,14–16} This requires the use of bioorthogonal chemistry¹⁷ which was developed mainly for proteins and carbohydrates,^{3–7,18} and has been very recently applied for nucleic acids, too. However, DNA and RNA chemistries are different from protein and carbohydrate chemistry, and bioorthogonal labelling chemistry cannot be simply transferred from proteins over carbohydrates to DNA and RNA.^{19,20}

“Click” chemistry²¹ is considered to be biocompatible, if the reactions do not require toxic catalysts, organic solvents, high temperatures or high pressure.²² Cycloadditions in general, and azide–alkyne cycloadditions in particular, are intensively used for nucleic acid labelling.^{23–25} One major reason is the advantage that both functional groups, azides and alkynes, are bioorthogonal and react chemoselectively with each other.²⁶ Since the early discovery by Huisgen,²⁷ the azide–alkyne cycloaddition, however, can only be used for biolabelling in the presence of a Cu(I) catalyst that accelerates this reaction such that it efficiently works at biological temperature.^{28,29} Chelating Cu(I) ligands, like tris(benzyltriazolylmethyl)-amine and water-soluble derivatives, suppress Cu(I)-induced oxidative DNA and RNA damages.^{30,31} The cytotoxicity of Cu(I)

salts is, however, a principal problem for labellings in the cellular environment. This limits the applicability of this reaction to fixed cells (*vide infra*). There are rare examples of protein labellings in living cells;^{32–34} to the best of our knowledge, there is no published example of a Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) for nucleic acid labelling in living cells. Among the copper-free click-type reactions, there are currently three main reactions used for nucleic acid labelling: (i) the ring-strain promoted azide–alkyne cycloadditions (SPAAC),³⁵ (ii) the “photoclick” 1,3-dipolar cycloadditions (PC)³⁶ and (iii) the Diels–Alder reactions with inverse electron demand (IEDDA).^{5–7} According to the literature, these three reaction types are considered to be bioorthogonal.³⁷ The available building blocks for these reactions were summarized recently by us^{19,20} and others.^{38,39} Herein, we review the labelling of DNA and RNA in the cellular environment of fixed (*in situ*) or living cells (*in vivo*) by the use of bioorthogonal cycloaddition chemistry. Additionally, we review the current status of orthogonal dual and triple labelling of DNA and RNA in the test tube (*in vitro*) to demonstrate their potential for future applications *in situ* or *in vivo*.

Discussion

Labelling of presynthesized reactive oligonucleotides inside cells

Postsynthetic labellings of reactive oligonucleotides^{18,40} are performed inside cells to check if the applied bioconjugation chemistry is indeed bioorthogonal. Such reactive nucleic acids were prepared by two major strategies (Fig. 1). (i) DNA and RNA was synthesized on solid phase (SPS) using a synthesizer and phosphoramidites with reactive groups (RG) as building blocks.^{41,42} Using this method, the sequence and the number of incorporated building blocks with reactive groups was controlled by the sequential synthesis. The reactive groups must be stable during DNA or RNA synthesis which includes strong acids for detritylation, strong oxidizers to convert the P(III) building blocks into P(V) and strong bases during workup. Unfortunately, not all bioorthogonally reactive groups withstand these conditions. For instance, tetrazines are highly reactive groups for IEDDA reactions, but are not stable enough for DNA and RNA solid-phase chemistry.⁴³ (ii) The enzymatic approach is a milder strategy to prepare DNA and RNA because it is performed in aqueous solution at neutral pH and without harsh chemicals.⁴⁴ 2'-Deoxyribonucleotide (dNTPs) and ribonucleoside triphosphates (NTPs) are the building blocks and have to be prepared as substrates for DNA and RNA polymerases.⁴⁵ Primer extension (PEX) or other enzymatic oligonucleotide extensions (EX) yield shorter oligonucleotides,⁴⁶ and PCR amplifies longer pieces of DNA. If a modified dN*TP (e.g. dA*TP) with a reactive group is mixed with the other three unmodified dNTPs (e.g. dGTP, dCTP and TTP) the reactive group is incorporated at every site where the corresponding dNTP is inserted by the enzyme. This multilabelling with reactive groups may be a disadvantage of this way of DNA



Hans-Achim Wagenknecht

Hans-Achim Wagenknecht studied chemistry in Freiburg (Germany), obtained his diploma in 1995 (glycosidase inhibitors, Jochen Lehmann), got his doctoral degree in 1998 in bioorganic chemistry (porphyrine enzyme models, Wolf-D. Woggon) in Basel (Switzerland) and worked as postdoc with Jacqueline K. Barton at Caltech (USA). His independent career started in 2000 in Munich (Germany). In 2003, he obtained the habilitation (charge transfer in

synthetically modified DNA) and in 2005 he got a professorship at Regensburg (Germany). Since 2010 he holds the chair for organic chemistry at the Karlsruhe Institute of Technology (KIT, Germany). His research is focused on bioorganic chemistry with nucleic acids and peptides, fluorescent imaging, DNA architectonics, photochemistry and chemical photocatalysis.



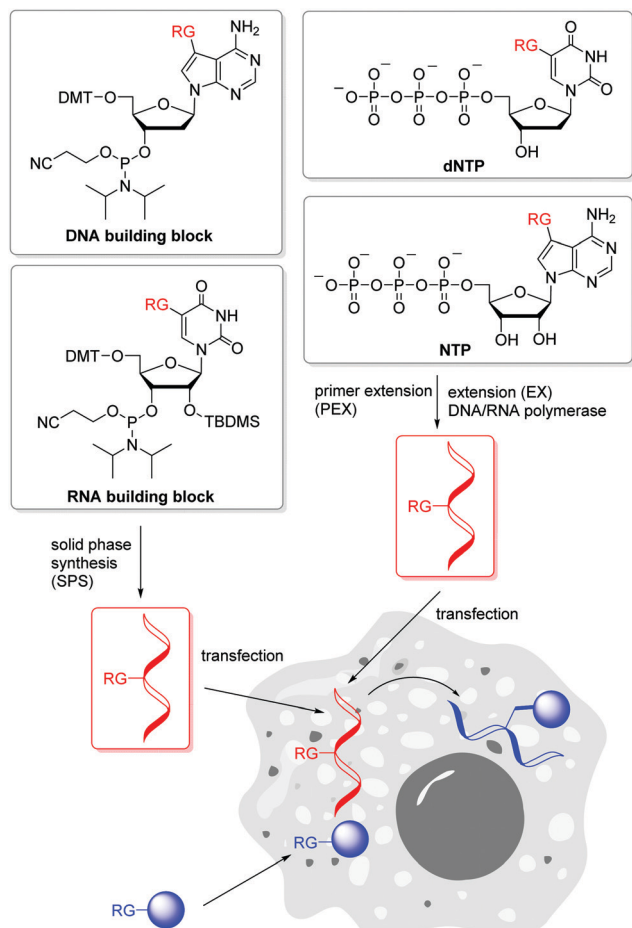


Fig. 1 Incorporation of 2'-deoxyribonucleotides and ribonucleotides with reactive groups (RG) into DNA and RNA (marked in red) for intracellular transport and subsequent postsynthetic labelling with reactive counter-parts (marked in blue) inside cells.

and RNA preparation due to its inhibitory effect on polymerases and the lack of control on the locations.

The SPAAC is an important alternative for the copper(i)-catalyzed reaction ($k_2 = 10\text{--}200\text{ M}^{-1}\text{ s}^{-1}$),⁴⁷ although the second rate order constants for conventional cyclooctynes are significantly lower ($k_2 = 0.0012\text{ M}^{-1}\text{ s}^{-1}$).^{16,48} The reaction kinetics were improved by increased ring strain. One of the most reactive cyclooctynes is biarylazacyclooctyne (BARAC, $k_2 = 0.96\text{ M}^{-1}\text{ s}^{-1}$),⁴⁹ but it is also quite lipophilic.⁵⁰ The carboxymethylmonobenzocyclooctyne (COMBO) is an alternative and very promising bioorthogonally reactive cyclooctyne that was invented by Kele *et al.* It is much smaller than the dibenzannulated cyclooctynes, like BARAC, and thus less lipophilic, but shows similar reactivity ($k_2 = 0.8\text{ M}^{-1}\text{ s}^{-1}$).⁵¹ We synthesized 2'-deoxyuridines as new DNA building blocks with the COMBO moiety attached to the 5-position using a rigid ethynyl linker⁵² or the flexible propyl linker.⁵³ The DNA building block **1** (Fig. 2) was chemically incorporated into oligonucleotides using SPS. HeLa cells were transfected with the synthetic DNA modified with **1** and were treated by azide-modified dyes.⁵³ The azide group quenches the fluorescence of the coumarin chromophore.

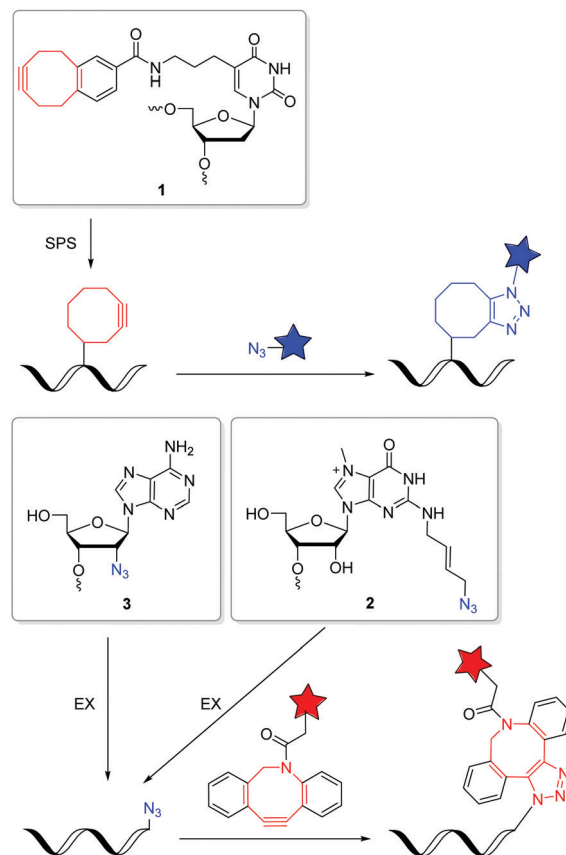


Fig. 2 DNA and RNA building blocks **1–3** for labelling by SPAAC in the cellular environment.

The azide moiety gets converted into the triazolyl moiety by the SPAAC with the modified DNA inside the living HeLa cells, and thus the fluorescence of the dyes lights up. Confocal microscopy revealed specific DNA staining in the nuclei. In a different approach, Rentmeister *et al.* modified the 5'-cap of the eGFP mRNA *in vitro* with the azide-RNA building block **2** using the corresponding nucleoside triphosphate and the methyltransferase Ecm1.⁵⁴ Mammalian cells were transfected with these azide-modified RNA and then reacted with BARAC-modified labels. After the in-cell SPAAC, the cells were fixed for better imaging. Alternatively, the poly(A) tail of mRNA could be labelled in a chemoenzymatic extension approach using the triphosphate of RNA building block **3** and yeast poly(A) polymerase.⁵⁵ In contrast to the previous approach with the 5'-cap, the enzyme installs multiple building blocks **3** to the eGFP mRNA. Confocal imaging of HeLa cells revealed that such poly(A) tail-modified mRNA is active for translation. Taken together these tools for intracellular labelling of mRNA are extremely important for studying subcellular localization and dynamic processes of target mRNAs in the future.

The iEDDA reactions between 1,2,4,5-tetrazines and strained alkenes or alkynes are currently the fastest copper-free alternatives for bioorthogonal labelling.^{56–58} Another advantage of this type of reactions is that the first cycloaddition is followed by an irreversible nitrogen release in a retro-Diels–Alder

reaction of the bicyclic intermediates to dihydropyridazines (with alkenes) or pyridazines (with alkynes). The iEDDA reactivity strongly depends on the substituents at the 1,2,4,5-tetrazines.^{59–62} The major problem for nucleic acid labelling by using 1,2,4,5-tetrazines as reactive groups, however, is the general instability towards the harsh and, in particular, basic conditions of the SPS,⁴³ but also towards the enzymatic DNA preparation by PEX.²⁰ There are only very few examples of 1,2,4,5-tetrazine-modified DNA and RNA in literature.^{20,43,63} To avoid this general problem, the alternative strategy is to incorporate the strained alkenes and alkynes into nucleic acids. In particular norbornenes,⁶⁴ bicyclo[6.1.0]nonynes (BCN),⁶⁵ *trans*-cyclooctenes (TCO)⁶⁶ and 1-methylcyclopropenes (MCP)⁶⁷ were established as functional groups for the iEDDA labelling of biomolecules, at first in proteins and later also in nucleic acids. However, there are only a few examples in literature for this type of labelling with presynthesized DNA or RNA in the cellular environment. Kath-Schorr *et al.* prepared norbornene-modified RNA.⁶⁸ The RNA building block 4 (Fig. 3) was incorporated at defined positions in predefined sequences by SPS. Mammalian cells (NCI-H460) were transfected with this presynthesized siRNA and treated with various 1,2,4,5-tetrazine-fluorophore conjugates. Confocal microscopy revealed that the ATTO647-labelled antisense strand of the siRNA duplex

colocalizes with the iEDDA reaction product of the sense strand. We applied the DNA building block 5 with the small cyclopropene group attached to the 7-position of the 7-deaza-2'-deoxyadenosine core for primer extension experiments with standard DNA polymerases.⁶⁹ This enzymatically prepared DNA strands were transfected into HeLa cells and reacted with a 1,2,4,5-tetrazine-TAMRA dye. Similar to the previous approach, the successful iEDDA reaction inside living cells was demonstrated by the colocalization of the TAMRA dye with the fluorescein label that was attached to the primer. These results demonstrate that strained alkenes represent an important chemical tool for the detection and investigation of RNA and DNA functions in cells, because they minimize perturbations of RNA functions by a large fluorophore and postpone the labelling to the final imaging of the living cells. Devaraj *et al.* used a template-dependent chemical ligation of fluorogenic probes for the detection of nucleic acids. Two different DNA pieces were presynthesized; the first piece of DNA was labelled by a quenched 1,2,4,5-tetrazine-fluorophore at the 5'-end and the second one by the MCP-modified building block 6 at the 3'-end. The ligation of the two modified oligonucleotides and the subsequent fluorogenic turn-on took place exclusively in the presence of the complementary template, compared to just minimal background reactions in the absence of the template. The enforced proximity of both reaction partners for the iEDDA reaction increases the effective molarity to a 50–120 mM range, depending on the linkers. This system was used for the detection of DNA in mammalian cells.⁷⁰ In another approach, the dienophile was replaced by the 7-azabenzonorbornadiene building block 7 and the tetrazine by a fluorogenic tetrazine-BODIPY, which led to an increase in turnover amplification after template-dependent ligation. The modified probes were used for the detection of the miRNA template mir-21 inside human cancer cell lines.⁶³

Metabolic labelling with reactive nucleosides and nucleotides

Metabolic labelling (ML) of nucleic acids focuses on the uptake of reactive nucleosides^{71,72} as well as nucleoside mono-,⁷³ or triphosphates⁷⁴ by living cells. After endogenous, enzymatic conversion into active nucleotides, they can be incorporated into nascent nucleic acids by DNA or RNA polymerases through successive rounds of replication or transcription, respectively. The provided label can postsynthetically be detected *via* a bioorthogonal reaction with a fluorescent reporter molecule (Fig. 4).

The incorporation of modified nucleosides into DNA and RNA is restricted by the acceptance of the nucleoside kinases. These enzymes are highly selective for the natural substrates due to the specific structures of active centres and the binding contacts. This initial phosphorylation step to the nucleoside monophosphates yield the building blocks for DNA and RNA polymerases. There are some approaches to overcome this limitation. The crystal structure of the uridine-cytidine kinase revealed an open structure at the C5 position.⁷² Likewise, the adenosine kinase showed cavernous open spaces at the C2, N6 and N7 positions.⁷² A modification at one of these sites can

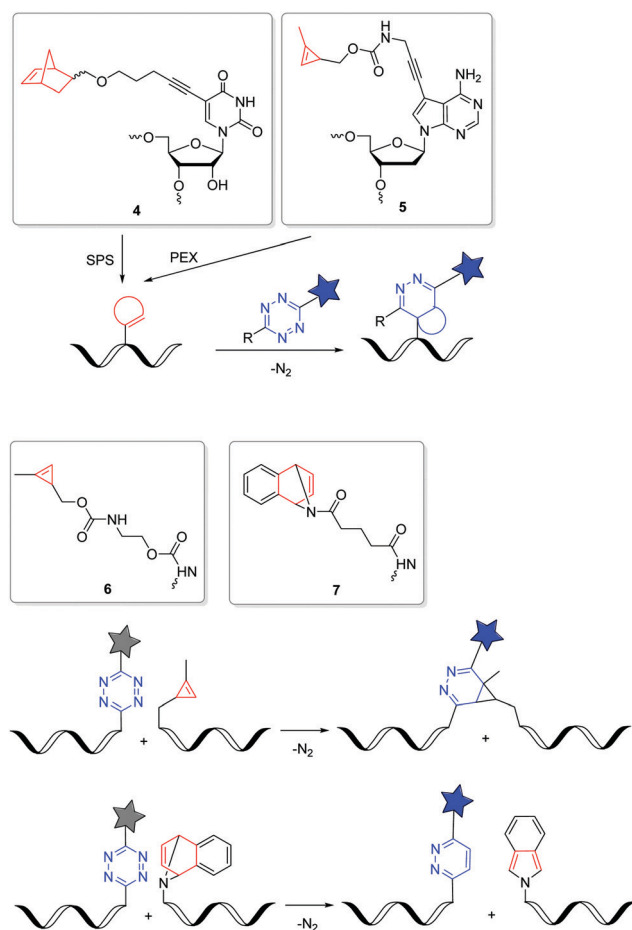


Fig. 3 DNA and RNA building blocks 4–7 for labelling by iEDDA reactions in the cellular environment.



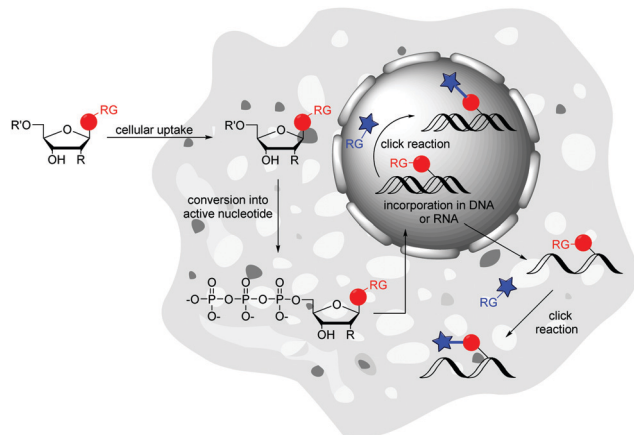


Fig. 4 Principle of metabolic labelling (ML) of nucleic acids: cellular uptake of reactive nucleosides or nucleotides, which are endogenous converted into active nucleotides and thus can be incorporated into DNA or RNA. Detection is followed by "click" reaction with fluorescent reporter molecule.

increase the acceptance of the nucleoside.^{72,75} Luedtke *et al.* worked with genetically modified cells that expressed a kinase of the herpes simplex virus that has reduced selectivity.⁷⁶ Another and less intrusive method is the use of so-called pronucleotides. These are nucleoside monophosphates in which the phosphate group is masked by two organic ester or amide groups to increase hydrophobicity and allows cell penetration. Once inside the cell, the chemical masks are cleaved by endogenous enzymes and the active nucleoside monophosphate is released.^{73,77} Common are the phosphoesters of 5-bisphaloyloxymethyl (POM) and 5-bis(4-acetoxybenzyl) (AB),⁷³ the phosphoramidites that are used for the "ProTide" protection groups⁷⁷ and cyclosal triesters.⁷⁸ To the best of our knowledge, there have been no publications on the introduction of nucleoside diphosphates into living cells, probably due to the inherent instability of the protected phosphate groups.⁷⁹ Also for nucleoside triphosphates there is a single publication by Zawada and Kraus, where a synthetic transporter complex is used for translocate nucleotides across the plasma membrane and release the active nucleotide into the cytoplasm.⁷⁴

It may be mentioned at this point that DNA and RNA polymerases also exhibit a certain substrate selectivity. This limitation is mainly explored for *in vitro* experiments to guide the incorporation of new nucleoside building blocks into DNA and RNA.^{80–82} Polymerase engineering yields advanced polymerases with improved functions by design, screening and targeted evolution. The intention is to improve their ability to incorporate, extend and replicate a wide variety of modified nucleotide analogues.⁸³ Recently, Spitale's group published the metabolic incorporation of exogenous uracil and uracil analogues into cellular RNA after specifically identifying that the uridine mono-phosphate synthase (UMPS) pathway and uracil phosphoribosyltransferase (UPRT) pathway yields the respective nucleotides. This provided a more complex understanding of the metabolic pathways responsible for the incorporation of bioorthogonal reporter molecules.⁸⁴

Subsequent to introducing the nucleoside into the cell and converting it to the active nucleotide, several strategies are now available to postsynthetically detect the labelled nucleic acids in the cellular environment. Due to the toxicity of some reactive nucleosides,^{76,85} the cytotoxic reaction conditions⁸⁶ and the cell preparations for labelling,⁷¹ as well as the limited membrane permeability of fluorescent reporter molecules,⁸⁷ postsynthetic labelling of nucleic acids was performed predominantly in fixed cells.

In 2008, Salic and Mitchison firstly published an *in vivo* strategy for the metabolic labelling of DNA using copper(I)-catalyzed azide–alkyne cycloaddition (CuAA). The incorporation of 5-ethynyl-2'-deoxyuridine **8** (Fig. 5) and its postsynthetic detection *via* reaction with fluorescent azides was a major breakthrough in the field of nascent DNA imaging, which was previously been done only by use of antibody staining.^{88,89} Despite the frequent use, **8** has been shown to be a highly toxic antimetabolite that can cause DNA strand damage leading to cell death.⁹⁰ Hence, further terminal alkyne-modified nucleosides were introduced, which had less toxic properties.⁹¹ In particular, the purine derivatives 7-deaza-7-ethynyl-2'-deoxyadenosine **9** and 7-deaza-7-ethynyl-2'-deoxyguanosine **10**⁹² as well as the arabinosyl derivative **11** of 5-ethynyl-2'-deoxyuridine published by Luedtke *et al.* showed lower toxicity in cell experiments.⁹³ Thus, these analogues were better adapted for long-term *in vivo* incubation. As an azide modified nucleoside, 5-(azidomethyl)-2'-deoxyuridine **12** was shown to be a viable alternative for postsynthetic labelling by CuAAC.⁷⁶

A copper-free alternative was recently published by Luedtke *et al.*, which was also the first time ever that a SPAAC was

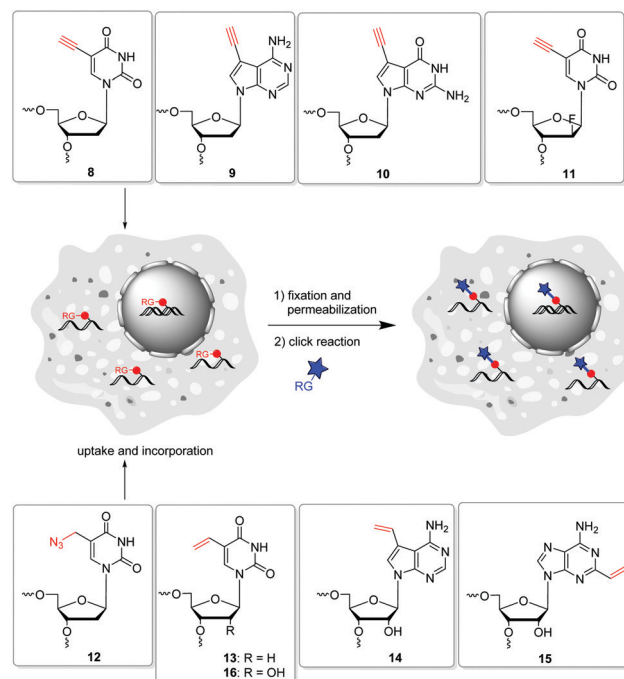


Fig. 5 Metabolic labelling of DNA with the modified building blocks **8–13** and of RNA with **14–16**.



applied for metabolic labelling of nucleic acids and as an *in vivo* experiment.⁹⁴ Before this approach, SPAACs were restricted to *in vitro* experiments due to the limited water solubility and poor uptake of the reactants.^{48,95} Luedtke's three-component "double click" strategy used a cationic Sondheimer diyne derivative for noncovalent intercalation into double-stranded DNA.⁹⁶ This creates a spatial proximity to the previously metabolically incorporated azide-modified nucleosides and yields efficient SPAACs. The resulting highly strained alkyne products then react in a second SPAAC with an azide, tagged to a fluorescent dye.

Another copper-free method introduced by Luedtke's group is based on iEDDA chemistry of 1,2,4,5-tetrazines and electron-rich dienophiles.⁵⁸ The reaction offers some major advantages: (i) it does not require a catalyst, (ii) it is irreversible due to the release of nitrogen, and (iii) it is compatible with cell media and therefore qualified for biological applications. 5-Vinyl-2'-deoxyuridine **13** was applied for metabolic labelling because it could successfully be incorporated into nascent DNA and detected by the reaction with a tetrazine-modified TAMRA dye. Based on this strategy, the incorporation of other vinyl-modified nucleosides, including 7-deaza-7-vinyl-adenosine **14** and 2-vinyl-adenosine **15** was also demonstrated for the metabolic labelling of RNA.^{72,97}

However, only a few of the above described methods for metabolic labelling of nucleic acids have the potential for labelling and detection in living cells. Imaging in fixed cells only provides information by a snapshot of living cells and is additionally affected by artefacts often caused by the fixation process.⁹⁸ In contrast, live cell imaging allows the monitoring of many dynamic intracellular processes in real time, which results in advanced kinetic and spatial information.^{99,100}

The CuAAC is highly restricted for the use in living systems by the intrinsic cytotoxicity of copper, causing oxidative DNA damage resulting in cell death in a short time.^{86,101} Where copper is no longer a problem in the SPAAC reaction, the restriction here is the limited cell permeability of the reaction partners. Due to the very bulky modifications, the acceptance of the kinases is an additional impediment for this reaction type.^{102,103} These limitations do not apply for metabolic labelling by iEDDA reaction. The vinyl-modified nucleosides are small enough to be taken up by the cell. They are accepted by the kinases, incorporated into nascent nucleic acids and do not show any significant cytotoxic properties. The postsynthetic labelling reaction takes place under physiological conditions. The major limiting factor is the essential denaturation of the DNA with 2 M HCl to allow reaction with the fluorescent tetrazine-dye. This prevents the detection of native DNA in the well preserved cellular environment, as well as the application in living systems.⁷¹ With regard to these issues, the following approach is promising; not an iEDDA reaction, but a hetero-Diels-Alder reaction was used by Tang *et al.* for labelling DNA. They demonstrated the metabolic incorporation of vinyl thioether thymidine as dienophile to enable hetero-Diels-Alder cycloaddition with *o*-quinolinone quinone methide (oQQF) under physiological conditions. The major advantage of this concept is that there is no need to denature the cellular DNA by HCl. However, labelling with the fluorescent oQQF was only performed in fixed cells.¹⁰⁴

Due to these non-biocompatible reaction conditions and preparations, the detection of labelled nucleic acids is still lacking usability in living systems. Another factor is the fluorescent reporter molecule which is used. While there is no problem with the availability of cell permeable "clickable" fluorescent dyes, which are most organic fluorophores that can be synthesised or purchased, it is more a problem to remove unbound dye from the inside of cells.^{105–107} While in fixed cells unbound dye can simply be washed out, this is a major challenge in living cells where it accumulates and causing fluorescent background signal. This problem may be solved by fluorogenic probes.^{108,109} In particular azides¹¹⁰ and tetrazines^{7,111} functions quench the fluorescence of dyes.

To the best of our knowledge, there are so far two publications reporting the detection of nucleic acids in living cells and even in living mice. (i) Last year, Xu *et al.* described the metabolic labelling of RNA with 5-vinyluridine **16** and the subsequent iEDDA reaction with a functionalized tetrazine linked to a biotin moiety. Detection is followed by fluorescent streptavidin-Alexa 488 reporter. Due to the high affinity of biotin and streptavidin, **16** can be linked to other molecules carrying streptavidin. This method may be paving the way to experiments that explore the dynamic interactions of RNA with other molecules.¹¹² In a further step, **16** was used to visualize tumors in living mice. For this approach, a light-up strategy of the fluorescence reporter was used to eliminate the fluorescent background, since in living systems the unbound dye cannot be removed. The BODIPY-tetrazine derivative developed for this purpose is the profluorophore, which only develops a strong fluorescence signal through the iEDDA reaction with the vinyl function of the nucleoside.¹⁰⁶ The injected modified nucleoside was able to accumulate in tumour tissue due to the increased metabolic activity of the tumour cells. The detection was subsequently performed with a light-up strategy using the fluorogenic BODIPY-tetrazine dye, which could be specifically detected in living mice. (ii) The second example applies the PC reaction which is the 1,3-dipolar cycloaddition between nitrile imines, that are generated by photolysis of tetrazoles, and olefines. This type of photoreaction was discovered by Huisgen *et al.*¹¹³ However, high temperatures or alternatively the use of light in the UV-B range do not meet the criteria of bioorthogonal labelling. The group of Lin *et al.* synthesized modified diaryltetrazoles for protein labelling which can be photolysed by light in the UV-A range.¹¹⁴ In principle, PC-type modifications of nucleic acids should now be applicable even to living cells. To the best of our knowledge, however, there is only one example published by Zhang *et al.* The group applied 5-vinyl-2'-deoxyuridine as building block **13** for metabolic labelling of DNA. Live cell labelling of DNA was reported to be efficient and fluorogenic with a water-soluble tetrazole and upon irradiation by 350 nm light.¹¹⁵

Orthogonal dual and triple labelling *in vitro*

The field of dual or even triple bioorthogonality is particularly challenging, but highly desired for investigations of structure changes and dynamics, in particular with FRET-based imaging probes. To achieve selective simultaneous labelling, a mutual



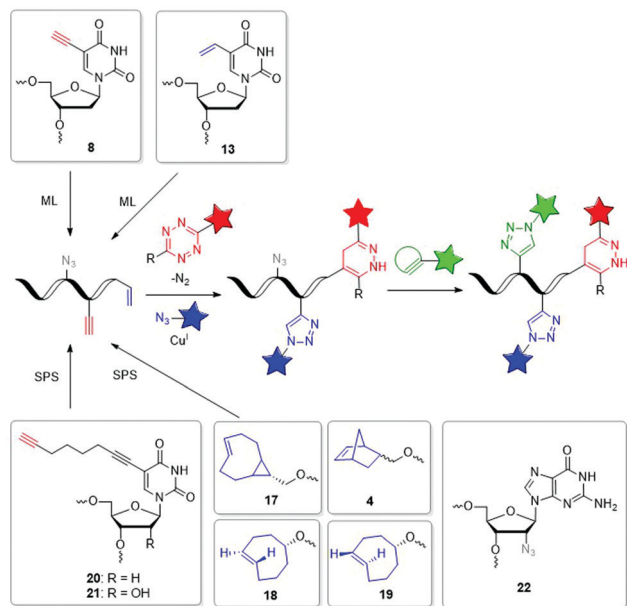


Fig. 6 Building blocks 17–22 such as 4, 8 and 13 for orthogonal dual and triple labelling of DNA and RNA by CuAAC, iEDDA and SPAAC.

orthogonality of the reactions must be given. Hildebrand *et al.* demonstrated for protein chemistry that TCO reacts with a 1,2,4,5-tetrazine in an iEDDA cycloaddition and an azide reacts with a dibenzocyclooctyne in a SPAAC and cross reactivity was excluded.¹¹⁶ iEDDA reactions¹¹⁷ with strained alkenes, in particular TCO and MCP, are orthogonal to SPAAC, but BCNs show cross reactivity.^{37,118} While some dual labelling schemes have been investigated and established for proteins^{119–122} and carbohydrates,¹²³ also a few can be found for nucleic acids (Fig. 6 and 7).^{71,124–126} Luedtke *et al.* synthesized the 5-vinyl-2'-deoxyuridine building block 13 for metabolic labelling by iEDDA cycloaddition and the 5-ethynyl-2'-deoxyuridine building block 8 by CuAAC labelling and evidenced their chemical orthogonality on fixed HeLa cells using two different fluorescent dyes.⁷¹ Jäschke *et al.* introduced the more reactive

dienophile modifications into DNA. The TCO-bearing phosphoramidites 17–19 were successfully attached to the 5'-end of DNA by SPS, and react with dansyl-modified 1,2,4,5-tetrazines.¹²⁴ Together with the alkyne-bearing building block 20 and an azide as reactive partner for the CuAAC dual labelling of DNA was realized in a one-pot reaction. This reaction combination was extended in another approach for efficient triple labelling of DNA and RNA by using the SPAAC for an additional level of orthogonality.¹²⁵ For the iEDDA cycloaddition the norbornene-modified building block 4 was attached at the 5'-end of the oligonucleotides. Applications with comparatively much more reactive TCO- and BCN-modifications showed high yields of by-product, which were probably caused by the usually disfavored reactions between azides and these dienophiles. For the CuAAC reaction, building blocks 20 and 21 were incorporated by SPS into DNA or RNA, respectively, while the 2'-azide-modified guanosine building block 22 was used for SPAAC. This approach could allow to study individual molecules by single molecule FRET (smFRET) and offer valuable insights into folding dynamics and presence of multiple folding states.¹²⁵

Our group managed to incorporate the MCP-bearing 7-deaza-2'-deoxyadenosine building block 5 and the 1,2,4-triazine-modified 2'-deoxyuridine building block 23 simultaneously into DNA strands by PEX.¹²⁷ For the dual labelling, two different iEDDA reactions were used, which reacted in an orthogonal fashion with each other. As reactive partner for the 1,2,4-triazine group of 23 a BCN-modified dye and for the MCP group of 5 a 1,2,4,5-tetrazine-modified dye was chosen. Careful gel electrophoretic analysis revealed a dual and orthogonal labelling of the PEX DNA products. In the meantime, this mutual orthogonality of iEDDA reactions with 1,2,4-triazines and 1,2,4,5-tetrazines was also confirmed by Prescher *et al.*¹²⁸ Although so far only performed in the test tube, such multiple position-selective postsynthetic modifications of nucleic acids offer a useful tool for bioorthogonal labelling and enables multi-component nucleic acid imaging in cells in the future.

Conclusions

Bioorthogonal cycloaddition chemistry requires reactive groups incorporated into DNA or RNA. This can be achieved either as presynthesized oligonucleotides with reactive groups by solid-phase chemistry, by polymerase-assisted preparation methods, such as primer extension (and subsequent intracellular transport), or by metabolic labelling of nucleosides with reactive groups. Recently, three main “click”-type reactions were explored in the cellular environment. The Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) is limited to its use in fixed cells and has been extensively used in the context of metabolic labelling. The ring-strain promoted azide–alkyne cycloaddition (SPAAC) and the Diels–Alder reactions with inverse electron demand (iEDDA) are considered to be bioorthogonal and there are many available building blocks. Both reactions were explored as postsynthetic labelling methods in the cellular environment by presynthesized oligonucleotides or by metabolic incorporation into nascent

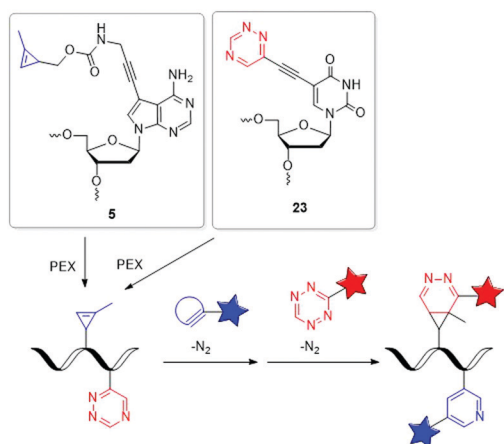


Fig. 7 Building blocks 5 and 23 for orthogonal dual labelling of DNA by two different iEDDA reactions.



DNA or RNA. The PC reaction is currently quite undeveloped with respect to potential for *in vivo* labelling. In particular, the spatiotemporal resolution that is associated in general with the use of light, could be a significant advantage for cellular applications in comparison to the more broadly applied SPAAC and iEDDA reactions. However, to ensure further promising and advanced approaches in particular for *in vivo* metabolic labelling of nucleic acids, the current limitations for bioorthogonal ligation have to be overcome, as they are not entirely suitable for living systems. Therefore, there is still a need to develop more flexible and biocompatible strategies to continuously expand the bioorthogonal toolbox for DNA and RNA labelling. Although multiple position-selective postsynthetic modification of nucleic acids can be performed in the test tube, it must be developed as a tool for multibioorthogonal labelling for nucleic acid imaging in cells in the future. Due to continuous research and the diversity of potential nucleoside analogues, the metabolic labelling of nucleic acids will offer important biological applications for imaging that have not yet been fully exploited.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (GRK 2039/2 and Wa 1386/15-2) and KIT is gratefully acknowledged.

Notes and references

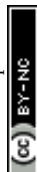
- V. Ntziachristos, Fluorescence Molecular Imaging, *Annu. Rev. Biomed. Eng.*, 2006, **8**, 1–33.
- R. Weissleder and V. Ntziachristos, Shedding light onto live molecular targets, *Nat. Med.*, 2003, **9**, 123–128.
- Y. Tian and Q. Lin, Fitness Factors for Bioorthogonal Chemical Probes, *ACS Chem. Biol.*, 2019, **14**, 2489–2496.
- S. Mayer and K. Lang, Tetrazines in Inverse-Electron-Demand Diels-Alder Cycloadditions and Their Use in Biology, *Synthesis*, 2017, 830–848.
- R. D. Row and J. A. Prescher, Constructing New Bioorthogonal Reagents and Reactions, *Acc. Chem. Res.*, 2018, **51**, 1073–1081.
- M. R. Karver, R. Weissleder and S. A. Hilderbrand, Bioorthogonal Reaction Pairs Enable Simultaneous, Selective, Multi-Target Imaging, *Angew. Chem., Int. Ed.*, 2012, **51**, 920–922.
- H. Wu and N. K. Devaraj, Advances in Tetrazine Bioorthogonal Chemistry Driven by the Synthesis of Novel Tetrazines and Dienophiles, *Acc. Chem. Res.*, 2018, **51**, 1249–1259.
- A. Hofer, Z. J. Liu and S. Balasubramanian, Detection, Structure and Function of Modified DNA Bases, *J. Am. Chem. Soc.*, 2019, **141**, 6420–6429.
- T. Tian, J. Wang and X. Zhou, A review: microRNA detection methods, *Org. Biomol. Chem.*, 2015, **13**, 2226–2238.
- J. J. Quinn and H. Y. Chang, Unique features of long non-coding RNA biogenesis and function, *Nat. Rev. Genet.*, 2016, **17**, 47–62.
- Y. Murakawa, M. Yoshihara, H. Kawaji, M. Nishikawa, H. Zayed, H. Suzuki, F. Consortium and Y. Hayashizaki, Enhanced Identification of Transcriptional Enhancers Provides Mechanistic Insights into Diseases, *Trends Genet.*, 2016, **32**, 76–88.
- M. T. Y. Lam, W. Li, M. G. Rosenfeld and C. K. Glass, Enhancer RNAs and regulated transcriptional programs, *Trends Biochem. Sci.*, 2014, **39**, 170–182.
- P. V. Chang and C. R. Bertozzi, Imaging beyond the proteome, *Chem. Commun.*, 2012, **48**, 8864–8879.
- P. Shieh and C. R. Bertozzi, Design strategies for bioorthogonal smart probes, *Org. Biomol. Chem.*, 2014, **12**, 9307–9320.
- K. Lang and J. W. Chin, Cellular Incorporation of Unnatural Amino Acids and Bioorthogonal Labeling of Proteins, *Chem. Rev.*, 2014, **114**, 4764–4806.
- C. P. Ramil and Q. Lin, Bioorthogonal chemistry: strategies and recent developments, *Chem. Commun.*, 2013, **49**, 11007–11022.
- P. Shieh and C. R. Bertozzi, Design strategies for bioorthogonal smart probes, *Org. Biomol. Chem.*, 2014, **12**, 9307–9320.
- I. Ivancová, D. Leone and M. Hocek, Reactive modifications of DNA nucleobases for labelling, bioconjugations and cross-linking, *Curr. Opin. Chem. Biol.*, 2019, **52**, 136–144.
- K. Krell, D. Harijan, D. Ganz, L. Doll and H.-A. Wagenknecht, Postsynthetic Modifications of DNA and RNA by Means of Copper-Free Cycloadditions as Bioorthogonal Reactions, *Bioconjugate Chem.*, 2020, (31), 990–1011.
- M. Merkel, S. Arndt, D. Ploschik, G. B. Cserép, U. Wenge, P. Kele and H.-A. Wagenknecht, Scope and Limitations of Typical Copper-Free Bioorthogonal Reactions with DNA: Reactive 2'-Deoxyuridine Triphosphates for Postsynthetic Labeling, *J. Org. Chem.*, 2016, **81**, 7527–7538.
- H. C. Kolb, M. G. Finn and K. B. Sharpless, Click Chemistry: Diverse Chemical Function From a Few Good Reactions, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021.
- C. J. Zeman, S. Kim, F. Zhang and K. S. Schanze, Direct Observation of the Reduction of Aryl Halides by a Photo-excited Perylene Diimide Radical Anion, *J. Am. Chem. Soc.*, 2020, **142**, 2204–2207.
- P. M. Gramlich, C. T. Wirges, A. Manetto and T. Carell, Postsynthetic DNA Modification through the Copper-Catalyzed Azide-Alkyne Cycloaddition Reaction, *Angew. Chem., Int. Ed.*, 2008, **47**, 8350–8358.
- A. H. El-Sagheer and T. Brown, Click chemistry with DNA, *Chem. Soc. Rev.*, 2010, **39**, 1388–1405.
- E. Paredes and S. R. Das, Click Chemistry for Rapid Labeling and Ligation of RNA, *ChemBioChem*, 2011, **12**, 125–131.
- M. F. Debets, C. W. J. V. D. Doelen, F. P. J. T. Rutjes and F. L. V. Delft, Azide: A Unique Dipole for Metal-Free Bioorthogonal Ligations, *ChemBioChem*, 2010, **11**, 1168–1184.



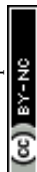
- 27 R. Huisgen, 1,3-Dipolar Cycloaddition. Past and Future, *Angew. Chem., Int. Ed. Engl.*, 1962, **2**, 565–598.
- 28 V. V. Rostovstev, L. G. Green, V. V. Fokin and K. B. Sharpless, A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective “Ligation” of Azides and Terminal Alkynes, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
- 29 C. W. Tornøe, C. Christensen and M. Meldal, Peptidetriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides, *J. Org. Chem.*, 2002, **67**, 3057–3064.
- 30 T. R. Chan, R. Hilgraf, K. B. Sharpless and V. V. Fokin, Polytriazoles as Copper(I)-Stabilizing Ligands in Catalysis, *Org. Lett.*, 2004, **6**, 2853–2855.
- 31 C. Besanceney-Webler, H. Jiang, T. Zheng, L. Feng, D. S. D. Amo, W. Wang, L. M. Klivansky, F. L. Marlow, Y. Liu and P. Wu, Increasing the Efficacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study, *Angew. Chem., Int. Ed.*, 2011, **50**, 8051–8056.
- 32 D. C. Kennedy, C. S. McKay, M. C. B. Legault, D. C. Danielson, J. A. Blake, A. F. Pegoraro, A. Stolor, Z. Mester and J. P. Pezacki, Cellular Consequences of Copper Complexes Used to Catalyze Bioorthogonal Click Reactions, *J. Am. Chem. Soc.*, 2011, **133**, 17993–18001.
- 33 V. Hong, N. F. Steinmetz, M. Manchester and M. G. Finn, Labeling Live Cells by Copper-Catalyzed Alkyne-Azide Click Chemistry, *Bioconjugate Chem.*, 2010, **21**, 1912–1916.
- 34 M. Yang, J. Li and P. R. Chen, Transition metal-mediated bioorthogonal protein chemistry in living cells, *Chem. Soc. Rev.*, 2014, **43**, 6511–6526.
- 35 J. J. Jewett and C. R. Bertozzi, Cu-free click cycloaddition reactions in chemical biology, *Chem. Soc. Rev.*, 2010, **39**, 1272–1279.
- 36 C. P. Ramil and Q. Lin, Photoclick chemistry: a fluorogenic light-triggered in vivo ligation reaction, *Curr. Opin. Chem. Biol.*, 2014, **21**, 89–95.
- 37 D. M. Patterson and J. A. Prescher, Orthogonal bioorthogonal chemistries, *Curr. Opin. Chem. Biol.*, 2015, **28**, 141–149.
- 38 M. Hock, Enzymatic Synthesis of Base-Functionalized Nucleic Acids for Sensing, Cross-linking, and Modulation of Protein-DNA Binding and Transcription, *Acc. Chem. Res.*, 2019, **52**, 1730–1737.
- 39 S. Kath-Schorr, Cycloadditions for Studying Nucleic Acids, *Top. Curr. Chem.*, 2016, **374**, 4.
- 40 W. Schmucker and H.-A. Wagenknecht, Organic Chemistry of DNA Functionalization; Chromophores as DNA Base Substitutes versus DNA Base/2'-Modifications, *Synlett*, 2012, 2435–2448.
- 41 C. B. Reese, Oligo- and poly-nucleotides: 50 years of chemical synthesis, *Org. Biomol. Chem.*, 2005, **3**, 3851–3868.
- 42 S. L. Beauchage and M. H. Caruthers, Deoxynucleoside phosphoramidites—A new class of key intermediates for deoxypolynucleotide synthesis, *Tetrahedron Lett.*, 1981, **22**, 1859–1862.
- 43 G. B. Cserép, O. Demeter, E. Bätzner, M. Kállay, H.-A. Wagenknecht and P. Kele, Synthesis and Evaluation of Nicotinic Acid Derived Tetrazines for Bioorthogonal Labeling, *Synthesis*, 2015, 2738–2744.
- 44 M. Hock, Enzymatic Synthesis of Base-Functionalized Nucleic Acids for Sensing, Cross-linking, and Modulation of Protein-DNA Binding and Transcription, *Acc. Chem. Res.*, 2019, **52**, 1730–1737.
- 45 J. Singh, A. Ripp, T. M. Haas, D. Qiu, M. Keller, P. A. Wender, J. S. Siegel, K. K. Baldrige and H. J. Jessen, Synthesis of Modified Nucleoside Oligophosphates Simplified: Fast, Pure, and Protecting Group Free, *J. Am. Chem. Soc.*, 2019, **141**, 15013–15017.
- 46 A. Hottin, K. Betz, K. Diederichs and A. Marx, Structural Basis for the KlenTaq DNA Polymerase Catalysed Incorporation of Alkene- versus Alkyne-Modified Nucleotides, *Chem. – Eur. J.*, 2017, **23**, 2109–2118.
- 47 K. Wang, A. Sachdeva, D. J. Cox, N. W. Wilf, K. Lang, S. Wallace, R. A. Mehl and J. W. Chin, Optimized orthogonal translation of unnatural amino acids enables spontaneous protein double-labelling and FRET, *Nat. Chem.*, 2014, **6**, 393–403.
- 48 K. Lang and J. W. Chin, Bioorthogonal Reactions for Labeling Proteins, *ACS Chem. Biol.*, 2014, **9**, 16–20.
- 49 J. C. Jewett, E. M. Sletten and C. R. Bertozzi, Rapid Cu-Free Click Chemistry with Readily Synthesized Biarylazacyclooctynes, *J. Am. Chem. Soc.*, 2010, **132**, 36878–36890.
- 50 M. F. Debets, J. C. M. v. Hest and F. P. J. T. Rutjes, Bioorthogonal labelling of biomolecules: new functional handles and ligation methods, *Org. Biomol. Chem.*, 2013, **11**, 6439–6455.
- 51 B. R. Varga, M. Kallay, K. Hegyi, S. Beni and P. Kele, A Non-Fluorinated Monobenzocyclooctyne for Rapid Copper-Free Click Reactions, *Chem. – Eur. J.*, 2012, **18**, 822–828.
- 52 C. Stubinitzky, G. B. Cserép, E. Bätzner, P. Kele and 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.
- 53 Á. Eördögh, J. Steinmeyer, K. Peewasan, U. Schepers, H.-A. Wagenknecht and P. Kéle, 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.
- 54 J. M. Holstein, L. Anhäuser and A. Rentmeister, Modifying the 5'-Cap for Click Reactions of Eukaryotic mRNA and To Tune Translation Efficiency in Living Cells, *Angew. Chem., Int. Ed.*, 2016, **55**, 10899–10903.
- 55 L. Anthäuser, S. Hüwel, T. Zobel and A. Rentmeister, Multiple covalent fluorescence labeling of eukaryotic mRNA at the poly(A) tail enhances translation and can be performed in living cells, *Nucleic Acids Res.*, 2019, **47**, e42.
- 56 R. A. Carboni and R. V. Lindsey, Reactions of Tetrazines with Unsaturated Compounds. A New Synthesis of Pyridazines, *J. Am. Chem. Soc.*, 1959, **81**(16), 4342–4346.
- 57 D. L. Boger and S. M. Sakya, Inverse electron demand Diels-Alder reactions of 3,6-bis(methylthio)-1,2,4,5-tetrazine. 1,2-Diazine introduction and direct implementation of a divergent 1,2,4,5-tetrazine.fwdarw. 1,2-Diazine.fwdarw.



- benzene (indoline/indole) Diels-Alder strategy, *J. Org. Chem.*, 1988, **53**(7), 1415–1423.
- 58 D. R. Soenen, J. M. Zimpleman and D. L. Boger, Synthesis and Inverse Electron Demand Diels–Alder Reactions of 3,6-Bis(3,4-dimethoxybenzoyl)-1,2,4,5-tetrazine, *J. Org. Chem.*, 2003, **68**(9), 3593–3598.
 - 59 J. Balcar, G. Chrisam, F. X. Huber and J. Sauer, Reaktivität von stickstoff-heterocyclen gegenüber cyclooctin als dienophil, *Tetrahedron Lett.*, 1983, **24**(14), 1481–1484.
 - 60 M. R. Karver, R. Weissleder and S. A. Hilderbrand, Synthesis and Evaluation of a Series of 1,2,4,5-Tetrazines for Bioorthogonal Conjugation, *Bioconjugate Chem.*, 2011, **22**(11), 2263–2270.
 - 61 D. L. Boger, R. P. Schaum and R. M. Garbaccio, Regio-selective Inverse Electron Demand Diels–Alder Reactions of N-Acyl 6-Amino-3-(methylthio)-1,2,4,5-tetrazines, *J. Org. Chem.*, 1998, **63**(18), 6329–6337.
 - 62 A. Hamasaki, R. Ducray and D. L. Boger, Two Novel 1,2,4,5-Tetrazines that Participate in Inverse Electron Demand Diels–Alder Reactions with an Unexpected Regioselectivity, *J. Org. Chem.*, 2006, **71**(1), 185–193.
 - 63 H. Wu, B. T. Cisneros, C. M. Cole and N. K. Devaraj, Bio-orthogonal Tetrazine-Modified Transfer Reactions Facilitate Reaction Turnover in Nucleic-Acid Templated Detection of MicroRNA, *J. Am. Chem. Soc.*, 2014, **136**, 17942–17945.
 - 64 A.-C. Knall, M. Hollauf and C. Slugovc, Kinetic studies of inverse electron demand Diels–Alder reactions (iEDDA) of norbornenes and 3,6-dipyridin-2-yl-1,2,4,5-tetrazine, *Tetrahedron Lett.*, 2014, **55**(34), 4763–4766.
 - 65 W. Chen, D. Wang, C. Dai, D. Hamelberg and B. Wang, Clicking 1,2,4,5-tetrazine and cyclooctynes with tunable reaction rates, *Chem. Commun.*, 2012, **48**(12), 1736–1738.
 - 66 R. Rossin, P. Renart Verkerk, S. M. van den Bosch, R. C. M. Volders, I. Verel, J. Lub and M. S. Robillard, *In Vivo* Chemistry for Pretargeted Tumor Imaging in Live Mice, *Angew. Chem., Int. Ed.*, 2010, **49**(19), 3375–3378.
 - 67 D. N. Kamber, L. A. Nazarova, Y. Liang, S. A. Lopez, D. M. Patterson, H.-W. Shih, K. N. Houk and J. A. Prescher, Isomeric Cyclopropenes Exhibit Unique Bioorthogonal Reactivities, *J. Am. Chem. Soc.*, 2013, **135**(37), 13680–13683.
 - 68 A. M. Pyka, C. Domnick, F. Braun and S. Kath-Schorr, Diels-Alder Cycloadditions on Synthetic RNA in Mammalian Cells, *Bioconjugate Chem.*, 2014, **25**, 1438–1443.
 - 69 D. Ploschik, F. Rönicke, H. Beike, R. Strasser and H.-A. Wagenknecht, DNA Primer Extension with Cyclopropenylated 7-Deaza-2'-deoxyadenosine and Efficient Bioorthogonal Labeling in Vitro and in Living Cells, *ChemBioChem*, 2018, **19**, 1949–1953.
 - 70 J. Šečut, J. Yang and N. K. Devaraj, Rapid oligonucleotide-templated fluorogenic tetrazine ligations, *Nucleic Acids Res.*, 2013, **41**(15), e148.
 - 71 U. Rieder and N. W. Luedtke, Alkene–Tetrazine Ligation for Imaging Cellular DNA, *Angew. Chem., Int. Ed.*, 2014, **53**(35), 9168–9172.
 - 72 M. Kubota, S. Nainar, S. M. Parker, W. England, F. Furche and R. C. Spitale, Expanding the Scope of RNA Metabolic Labeling with Vinyl Nucleosides and Inverse Electron-Demand Diels-Alder Chemistry, *ACS Chem. Biol.*, 2019, **14**, 1698–1707.
 - 73 M. Tera, S. M. K. Glasauer and N. W. Luedtke, In Vivo Incorporation of Azide Groups into DNA by Using Membrane-Permeable Nucleotide Triesters, *ChemBioChem*, 2018, **19**, 1939–1943.
 - 74 Z. Zawada, A. Tatar, P. Mocilac, M. Budesinsky and T. Kraus, Transport of Nucleoside Triphosphates into Cells by Artificial Molecular Transporters, *Angew. Chem., Int. Ed.*, 2018, **57**(31), 9891–9895.
 - 75 A. R. Van Rompay, A. Norda, K. Linden, M. Johansson and A. Karlsson, Phosphorylation of uridine and cytidine nucleoside analogs by two human uridine-cytidine kinases, *Mol. Pharmacol.*, 2001, **59**(5), 1181–1186.
 - 76 A. B. Neef and N. W. Luedtke, An Azide-Modified Nucleoside for Metabolic Labeling of DNA, *ChemBioChem*, 2014, **15**, 789–793.
 - 77 C. McGuigan, R. N. Pathirana, N. Mahmood, K. G. Devine and A. J. Hay, Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines which are resistant to the action of AZT, *Antiviral Res.*, 1992, **17**(4), 311–321.
 - 78 N. Huynh, C. Dickson, D. Zencak, D. H. Hilko, A. Mackay-Sim and S. A. Poulsen, Labeling of Cellular DNA with a Cyclosal Phosphotriester Pronucleotide Analog of 5-ethynyl-2'-deoxyuridine, *Chem. Biol. Drug Des.*, 2015, **86**(4), 400–409.
 - 79 H. J. Jessen, T. Schulz, J. Balzarini and C. Meier, Bio-reversible protection of nucleoside diphosphates, *Angew. Chem., Int. Ed.*, 2008, **47**(45), 8719–8722.
 - 80 K. Betz, F. Streckenbach, A. Schnur, T. Exner, W. Welte, K. Diederichs and A. Marx, Structures of DNA polymerases caught processing size-augmented nucleotide probes, *Angew. Chem., Int. Ed.*, 2010, **49**(30), 5181–5184.
 - 81 A. Hottin, K. Betz, K. Diederichs and A. Marx, Structural Basis for the KlenTaq DNA Polymerase Catalysed Incorporation of Alkene- versus Alkyne-Modified Nucleotides, *Chem. – Eur. J.*, 2017, **23**(9), 2109–2118.
 - 82 V. Raindlova, M. Janouskova, M. Slavickova, P. Perlikova, S. Bohacova, N. Milisavljevic, H. Sanderova, M. Benda, I. Barvik, L. Krasny and M. Hocek, Influence of major-groove chemical modifications of DNA on transcription by bacterial RNA polymerases, *Nucleic Acids Res.*, 2016, **44**(7), 3000–3012.
 - 83 D. Loakes and P. Holliger, Polymerase engineering: towards the encoded synthesis of unnatural biopolymers, *Chem. Commun.*, 2009, (31), 4619–4631.
 - 84 S. Nainar, B. J. Cuthbert, N. M. Lim, W. E. England, K. Ke, K. Sophal, R. Quechol, D. L. Mobley, C. W. Goulding and R. C. Spitale, An optimized chemical-genetic method for cell-specific metabolic labeling of RNA, *Nat. Methods*, 2020, **17**(3), 311–318.
 - 85 F. Kohlmeier, A. Maya-Mendoza and D. A. Jackson, EdU induces DNA damage response and cell death in mESC in culture, *Chromosome Res.*, 2013, **21**(1), 87–100.
 - 86 D. C. Kennedy, C. S. McKay, M. C. B. Legault, D. C. Danielson, J. A. Blake, A. F. Pegoraro, A. Stelow, Z. Mester



- and J. P. Pezacki, Cellular Consequences of Copper Complexes Used to Catalyze Bioorthogonal Click Reactions, *J. Am. Chem. Soc.*, 2011, **133**, 17993–18001.
- 87 C. W. Cunningham, A. Mukhopadhyay, G. H. Lushington, B. S. J. Blagg, T. E. Prisinzano and J. P. Krise, Uptake, distribution and diffusivity of reactive fluorophores in cells: implications toward target identification, *Mol. Pharming*, 2010, **7**(4), 1301–1310.
 - 88 A. Salic and T. J. Mitchison, A chemical method for fast and sensitive detection of DNA synthesis in vivo, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**(7), 2415–2420.
 - 89 R. M. Hamelik and A. Krishan, Click-iT assay with improved DNA distribution histograms, *Cytometry, Part A*, 2009, **75**(10), 862–865.
 - 90 H. Zhao, H. D. Halicka, J. Li, E. Biela, K. Berniak, J. Dobrucki and Z. Darzynkiewicz, DNA damage signaling, impairment of cell cycle progression, and apoptosis triggered by 5-ethynyl-2'-deoxyuridine incorporated into DNA, *Cytometry, Part A*, 2013, **83**(11), 979–988.
 - 91 L. Guan, G. W. van der Heijden, A. Bortvin and M. M. Greenberg, Intracellular detection of cytosine incorporation in genomic DNA by using 5-ethynyl-2'-deoxycytidine, *ChemBioChem*, 2011, **12**(14), 2184–2190.
 - 92 A. B. Neef, F. Samain and N. W. Luedtke, Metabolic labeling of DNA by purine analogues in vivo, *ChemBioChem*, 2012, **13**(12), 1750–1753.
 - 93 A. B. Neef and N. W. Luedtke, Dynamic metabolic labeling of DNA in vivo with arabinosyl nucleosides, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**(51), 20404–20409.
 - 94 M. Tera and N. W. Luedtke, Three-Component Bioorthogonal Reactions on Cellular DNA and RNA, *Bioconjugate Chem.*, 2019, **30**, 2991–2997.
 - 95 R. Chadwick, S. Gyzen, S. Liogier and A. Adronov, Scalable Synthesis of Strained Cyclooctyne Derivatives, *Synthesis*, 2014, 669–677.
 - 96 M. Tera, Z. Harati Taji and N. W. Luedtke, Intercalation-enhanced “Click” Crosslinking of DNA, *Angew. Chem., Int. Ed.*, 2018, **57**(47), 15405–15409.
 - 97 J. T. George and S. G. Srivatsan, Vinyluridine as a Versatile Chemoselective Handle for the Post-transcriptional Chemical Functionalization of RNA, *Bioconjugate Chem.*, 2017, **28**(5), 1529–1536.
 - 98 J. W. Lichtman and J.-A. Conchello, Fluorescence microscopy, *Nat. Methods*, 2005, **2**(12), 910–919.
 - 99 D. J. Stephens and V. J. Allan, Light microscopy techniques for live cell imaging, *Science*, 2003, **300**(5616), 82–86.
 - 100 L. Parhamifar, L. Wu, H. Andersen and S. M. Moghimi, Live-cell fluorescent microscopy platforms for real-time monitoring of polyplex-cell interaction: Basic guidelines, *Methods*, 2014, **68**(2), 300–307.
 - 101 G. R. Abel, Jr., Z. A. Calabrese, J. Ayco, J. E. Hein and T. Ye, Measuring and Suppressing the Oxidative Damage to DNA During Cu(I)-Catalyzed Azide-Alkyne Cycloaddition, *Bioconjugate Chem.*, 2016, **27**(3), 698–704.
 - 102 N. J. Agard, J. A. Prescher and C. R. Bertozzi, A Strain-Promoted [3+2] Azide-Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems, *J. Am. Chem. Soc.*, 2004, **126**, 15046–15047.
 - 103 M. F. Debets, S. S. van Berkel, S. Schoffelen, F. P. Rutjes, J. C. van Hest and F. L. van Delft, Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition, *Chem. Commun.*, 2010, **46**(1), 97–99.
 - 104 A. Gubu, L. Li, Y. Ning, X. Zhang, S. Lee, M. Feng, Q. Li, X. Lei, K. Jo and X. Tang, Bioorthogonal Metabolic DNA Labelling using Vinyl Thioether-Modified Thymidine and o-Quinolinone Quinone Methide, *Chem. – Eur. J.*, 2018, **24**(22), 5895–5900.
 - 105 F. Doll, A. Buntz, A.-K. Späte, V. D. Schart, A. Timper, W. Schrimpf, C. R. Hauck, A. Zumbusch and V. Wittmann, Visualization of Protein-Specific Glycosylation Inside Living Cells, *Angew. Chem., Int. Ed.*, 2016, **55**, 2262–2266.
 - 106 J. C. T. Carlson, L. G. Meimetis, S. A. Hilderbrand and R. Weissleder, BODIPY-Tetrazine Derivatives as Superbright Bioorthogonal Turn-on Probes, *Angew. Chem., Int. Ed.*, 2013, **52**(27), 6917–6920.
 - 107 K. E. Beatty, J. D. Fisk, B. P. Smart, Y. Y. Lu, J. Szychowski, M. J. Hangauer, J. M. Baskin, C. R. Bertozzi and D. A. Tirrell, Live-cell imaging of cellular proteins by a strain-promoted azide-alkyne cycloaddition, *ChemBioChem*, 2010, **11**(15), 2092–2095.
 - 108 A. Nadler and C. Schultz, The Power of Fluorogenic Probes, *Angew. Chem., Int. Ed.*, 2013, **52**, 2408–2410.
 - 109 E. Kozma and P. Kele, Fluorogenic probes for super-resolution microscopy, *Org. Biomol. Chem.*, 2019, **17**, 215–233.
 - 110 A. Herner, I. Nikic, M. Kallay, E. A. Lemke and P. Kele, A new family of bioorthogonally applicable fluorogenic labels, *Org. Biomol. Chem.*, 2013, **11**, 3297–3306.
 - 111 A. Wiczorek, P. Werther, J. Euchner and R. Wombacher, Green- to far-red-emitting fluorogenic tetrazine probes – synthetic access and no-wash protein imaging inside living cells, *Chem. Sci.*, 2017, **8**, 1506–1510.
 - 112 H. S. Liu, T. Ishizuka, M. Kawaguchi, R. Nishii, H. Kataoka and Y. Xu, A Nucleoside Derivative 5-Vinyluridine (VrU) for Imaging RNA in Cells and Animals, *Bioconjugate Chem.*, 2019, **30**(11), 2958–2966.
 - 113 J. S. Clovis, A. Eckell, R. Huisgen and R. Sustmann, 1,3-Dipolare Cycloadditionen, XXV. Der Nachweis des freien Diphenylnitrilimins als Zwischenstufe bei Cycloadditionen, *Chem. Ber.*, 1967, **100**, 60–70.
 - 114 Y. Wang, W. J. Hu, W. Song, R. K. V. Lim and Q. Lin, Discovery of Long-Wavelength Photoactivatable Diaryl-tetrazoles for Bioorthogonal 1,3-Dipolar Cycloadditions, *Org. Lett.*, 2008, **10**, 3725–3728.
 - 115 Y. Wu, G. Guo, J. Zheng, D. Xing and T. Zhang, Fluorogenic “Photoclick” Labeling and Imaging of DNA with Coumarin-Fused Tetrazole in Vivo, *ACS Sens.*, 2019, **4**, 44–51.
 - 116 M. R. Karver, R. Weissleder and S. A. Hilderbrand, Bioorthogonal Reaction Pairs Enable Simultaneous, Selective, Multi-Target Imaging, *Angew. Chem., Int. Ed.*, 2012, **51**(4), 920–922.
 - 117 B. L. Oliveira, Z. Guo and G. J. L. Bernardes, Inverse electron demand Diels-Alder reactions in chemical biology, *Chem. Soc. Rev.*, 2017, **46**, 4895–4950.



- 118 A.-C. Knall and C. Slugovc, Inverse electron demand Diels-Alder (iEDDA)-initiated conjugation: a (high) potential click chemistry scheme, *Chem. Soc. Rev.*, 2013, **42**, 5131–5142.
- 119 T. Plass, S. Milles, C. Koehler, J. Szymański, R. Mueller, M. Wießler, C. Schultz and E. A. Lemke, Amino Acids for Diels-Alder Reactions in Living Cells, *Angew. Chem., Int. Ed.*, 2012, **51**(17), 4166–4170.
- 120 D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber and J. A. Prescher, Functionalized Cyclopropenes As Bioorthogonal Chemical Reporters, *J. Am. Chem. Soc.*, 2012, **134**(45), 18638–18643.
- 121 I. Nikić, T. Plass, O. Schraidt, J. Szymański, J. A. G. Briggs, C. Schultz and E. A. Lemke, Minimal Tags for Rapid Dual-Color Live-Cell Labeling and Super-Resolution Microscopy, *Angew. Chem., Int. Ed.*, 2014, **53**(8), 2245–2249.
- 122 A. Sachdeva, K. Wang, T. Elliott and J. W. Chin, Concerted, Rapid, Quantitative, and Site-Specific Dual Labeling of Proteins, *J. Am. Chem. Soc.*, 2014, **136**(22), 7785–7788.
- 123 A. Niederwieser, A.-K. Späte, L. D. Nguyen, C. Jüngst, W. Reutter and V. Wittmann, Two-Color Glycan Labeling of Live Cells by a Combination of Diels-Alder and Click Chemistry, *Angew. Chem., Int. Ed.*, 2013, **52**(15), 4265–4268.
- 124 J. Schoch, M. Staudt, A. Samanta, M. Wiessler and A. Jäschke, Site-Specific One-Pot Dual Labeling of DNA by Orthogonal Cycloaddition Chemistry, *Bioconjugate Chem.*, 2012, **23**(7), 1382–1386.
- 125 M.-L. Winz, E. C. Linder, J. Becker and A. Jäschke, Site-specific one-pot triple click labeling for DNA and RNA, *Chem. Commun.*, 2018, **54**(83), 11781–11784.
- 126 P. V. Chang and C. R. Bertozzi, Imaging beyond the proteome, *Chem. Commun.*, 2012, **48**, 8864–8879.
- 127 U. Reisacher, D. Ploschik, F. Röncke, G. B. Cserép, P. Kele and H.-A. Wagenknecht, Copper-free dual labeling of DNA by triazines and cyclopropenes as minimal orthogonal and bioorthogonal functions, *Chem. Sci.*, 2019, **10**(14), 4032–4037.
- 128 D. N. Kamber, Y. Liang, R. J. Blizzard, F. Liu, R. A. Mehl, K. N. Houk and J. A. Prescher, 1,2,4-Triazines Are Versatile Bioorthogonal Reagents, *J. Am. Chem. Soc.*, 2015, **137**(26), 8388–8391.

