kelep_189_24

Bioorthogonally modulated photoresponsive systems for chemical biology applications

by

Péter Kele

submitted in partial fulfillment of the requirements for the title, Doctor of the Hungarian Academy of Sciences

HUN-REN, Research Centre for Natural Sciences, Institute of Organic Chemistry

2024

kelep_189_24

"Luck is what happens **when preparation meets opportunity**"

- Seneca

TABLE OF CONTENTS

PREFACE

The quote "luck is what happens when preparation meets opportunity" is attributed to Seneca, although there is hardly any evidence that he ever said it. Although it may be interpreted differently by others, to me it suggests that circumstances are not the ones that control things that happen to us, researchers, or individuals. Rather, it is ourselves, our talent and our mindset formed by learning and working hard that make us see – or not see – the potential in the circumstances. An important addition to this is that we need to seize as many of these circumstances as possible, rather than passively waiting for them to happen.

It is also of great importance to give credit to other people who are likely to contribute our success. Kindly pushed toward chemistry by an influential chemistry teacher, **Ilona Kozári**, I ended up at the Eötvös Loránd University as a biology and chemistry major, where I had a lot of circumstances to seize. The first of these was probably when I was looking for a laboratory to carry out research in my junior year. Under the thoughtful and sometimes strict guidance of **Prof. Ferenc Sebestyén**, I was introduced to fluorescence, a phenomenon that fascinated and captivated me and has held me ever since. Also, these were the years when I initiated my first collaborations in my own ways, probably triggered by the recognition of my own limitations. Determined by my shared interest toward biology and chemistry, which guided me to the interdisciplinary field of chemical biology, collaborative work became a foundation of my research. Venturing to do a PhD in the US was probably also a chase after further circumstances. Under the friendly, indulgent and permissively loose supervision of **Prof. Roger M. Leblanc** at the University of Miami, I had the opportunity to try myself as an individual researcher. Inspired by the highly inderdisciplinary environment, I was allowed to come up with ideas and conduct the related research. Returning to Hungary following my PhD placed me to an entirely different environment as a post-doctoral fellow. The lesson here, learned from **Prof. András Kotschy** was, how to ask questions from different perspectives, and how those questions may lead to brilliant ideas. As a Humboldt fellow, I had the luck to meet my friend and "Doktorvater" at the University of Regensburg. **Prof. Otto S. Wolfbeis** took this role seriously. The term "Doktorvater" reflects the obligation of a supervisor to support the career of the younger ones in the best possible way. He gave me a very good piece of advice of finding a research topic which can be linked to my name. Further to this, He introduced me to click chemistry, which led directly to bioorthogonal chemistry. Advice taken, Otto. The first time I read about bioorthogonal chemistry I knew this was a good fit to me and hopefully I was a good match to bioorthogonal chemistry as well.

Besides the great responsibility, the "Lendület" Award of the Hungarian Academy of Sciences also provided me with great opportunity. Starting my independent research carreer in such a supportive

environment led to the foundation of the Chemical Biology Research Group, which conducts research topics that combines bioorthogonality and photoresponsivity. I intend to use this latter term as it combines fluorescent markers with photolabile protecting groups, both responding to light albeit in different ways. By becoming a supervisor, I myself became responsible for passing on all the knowledge I had received to my students and colleagues. Here is a list of the key players who are currently or previously accompany me on this road as students or collegues and created a supportive and highly inspiring atmosphere, where friendly or passionate discussions foster ideas and research directions: **Krisztina "MegaStokes" Nagy**, my first PhD student, **Krisztián "sequential-click" Lőrincz**, who could really take everything easy, **András "fluorogenic" Herner**, who often challenged my patience with his thoroughness, **Balázs "COMBO" Varga**, who also challenged me albeit in a different way, **Gergely "one ring to rule them all" Cserép**, who stands by me for the longest time, **Eszter "DOTCO" Kozma**, who taught us different perspectives, **Orsolya "double the quencher" Demeter**, who raised ironic humor in the lab to a next level, **Bianka "the artist" Söveges**, whose kindness held me back to be too harsh on others, **Gergely "cicanine" Knorr**, who taught us that molecules are smart enough to find each other in a flask, **Evelin "cassette" Albitz**, who kept us organized, **Alexandra "blushing" Egyed**, who taught us a lesson in perseverance, **Dóra "dancing queen" Kern**, who can hand-draw print-ready chemical structures, **Orsolya "Snow White" Ember**, who stands my "humor" with a smile, **Tibor "shift that wavelength Tibike" Molnár**, who quietly does what he needs to and **Boldizsár "unheard grant" Boczán**, who is aware of each and every scholarship within the Carpathian Basin. **Attila "magic hand" Kormos**, who knits molecules or dachshunds, **Krisztina "cell lab" Németh**, who tries to make me be more realistic, **Ágnes "FAST" Szatmári**, who would have never said such words before and **Márton "boss, it's a bulls*t" Bojtár**, who is the most passionate chemist I have ever met. Unfortunately, I can't list all the BSc and MSc students that were around in the lab and made their own contributions to our research. Thank you all, for all the blood, sweat, tears, joy and happiness! I also thank all my collaboration partners and friends, **Krisztina Hegyi**, **Mihály Kállay, Gábor Mező, György Török, Edward A. Lemke and Hans-Achim Wagenknecht**.

I want to express my gratitude to my wife, **Emese**, my most critical supporter, to whom I owe more than I can list. I thank my kids, **János**, **Mihály** and **Anna**, who remind me the real values of life and who teach me how to explain the big questions of nature, at least to the best of my competence. Finally, I have to acknowledge my **mother** for providing me the opportunity to study and my **father-in-law** for the beer.

Whatever this term means, **I feel lucky** to know all of you.

INTRODUCTION TO BIOORTHOGONAL CHEMISTRY AND PHOTORESPONSIVE COMPOUNDS

Bioorthogonal chemistry

The use of chemically and biologically inert, non-perturbing, non-interfering, non-natural chemical handles to carry out selective chemical reactions in live organisms promoted the Nobel-prize winning conceptualization of *bioorthogonal chemistry*, a term coined by Carolyn R. Bertozzi.[1,2,3] Bioorthogonal chemical transformations can be considered as the biocompatible subset of click-chemistry. Effectively addressing the ultimate challenge of selective and site-specific manipulation of biomolecules in their natural context, bioorthogonal reactions have become indispensable tools to install e.g., small organic, minimally perturbing markers onto various cellular targets. As such, bioorthogonal reactions found valuable applications in the field of Chemical Biology, a scientific discipline that aims to understand biological systems through precise small molecular manipulations.[4] Following the postulation of the bioorthogonal concept a set of reactions with the potential to be applicable in living systems were identified. Some consider a wider selection of bioorthogonal reactions as bioorthogonal, e.g. aldehyde/ketone-amine ligations, I limit my discussion to chemistries that either use purely non-natural functions or non-natural combinations of natural functions (e.g. Cys_4-tag) to ensure selective ligation in live systems.

Staudinger-Bertozzi ligation[3]

Triaryl-phosphines harboring a rationally positioned ester function as an electrophilic trap and organic azides are more or less stable functions in a biological environment and indifferent to the presence of natural functions. Such phosphines react readily although at a limited rate with azides according to the meachanism of the Staudinger reduction (Figure 1). The primary adduct is then rearranged internally leading to the formation of a stable amide bond. Though this transformation is quite slow (Figure 1) and some of the reagents is lost due to spontaneous oxidation of the phosphines, it is credited for its full biocompatibility, selectivity and its potential to construct fluorogenic probes.

Photoclick reaction[5]

UV-light triggered generation of reactive nitrile-imines from diaryl tetrazoles or diaryl sydnones can also be considered as a bioorthogonal reaction as demonstrated by Lin et al. The generated reactive dipolar species undergoes rapid cycloaddition with olefins affording fluorescent pyrazoline derivatives (Figure 1). While the need for UV irradiation limits its use to a certain degree in live cells, the inherent fluorogenicity and photo-controllability, enabling spatial and temporal control

over the reaction, makes this transformation quite appealing. Besides the photoclick recations of tetrazoles, further light induced bioorthogonal reactions were also developed, however, these remained moderately significant.[6,7]

Azide-alkyne cycloadditions

Another 1,3-dipolar cycloaddition (CuAAC) also makes use of the small, stable, yet highly energetic azide group.[8] The highly valuable regioselective copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction of organic azides and terminal alkynes in the presence of Cu(I) catalyst gives rise to the formation of a stable, covalent 1,4-disubstituted triazole-linkage.[9,10] The need for copper catalyst, however, limits *in cellulo* or *in vivo* applicability of this reaction. Although successful attempts managed to somewhat mask the toxicity of the copper by applying appropriate ligand additives these approaches have not become popular.[11,12] An alternative and widespread approach that eliminates the need for toxic copper catalyst was introduced by Bertozzi and co-workers. In their pioneering work they demonstrated that introduction of strain into the alkyne reactant efficiently promotes the reaction to proceed at room temperature without any catalyst (Figure 1).[13,14] This approach eliminates the need for the metal catalyst although at a cost: the strain promoted azide-alkyne cycloaddition (SPAAC) proceeds at considerably slower rates than its copper catalyzed version. This latter issue was addressed by many laboratories, including ours, by developing more reactive cyclooctyne derivatives.[14] There are two major approaches to improve the reactivity of cyclooctynes towards azides: either lower the LUMO of the alkyne by the incorporation of strong electron-withdrawing groups (typically gem-difluoro substituents α to the alkyne, as in difluorocyclooctyne (DIFO) or further increase the ring strain (as in dibenzocyclooctyne (DIBO), and bicyclo[6.1.0]nonyne (BCN).

Inverse electron demand Diels-Alder reactions[15,16]

A real milestone was achieved in catalyst-free bioorthogonal ligations by revisiting the reactions of tetrazines and strained ring dienophiles. The strain promoted inverse electron demand Diels-Alder cycloaddition (iEDDA) reaction between these reactants really meets the need for demanding biological applications. Although synthesis of the reagents may require laborious synthetic work, tetrazines in combination with various strained dienophiles such as cyclopropenes, norbornenes, cyclooctynes and especially *trans*-cyclooctenes (TCOs) display the fastest rates of all bioorthogonal reactions (Figure 1). The reactivity of the tetrazines could be tuned either by reducing the steric bulk of the substituents or by increasing the electron withdrawing nature of substituents, however, for biological applications a trade-off should be made between reactivity and stability.[17] Most tetrazine appended probes apply either a 3-methyl-6-phenyl-1,2,4,5-tetrazine (Me-Tet) or a 3-

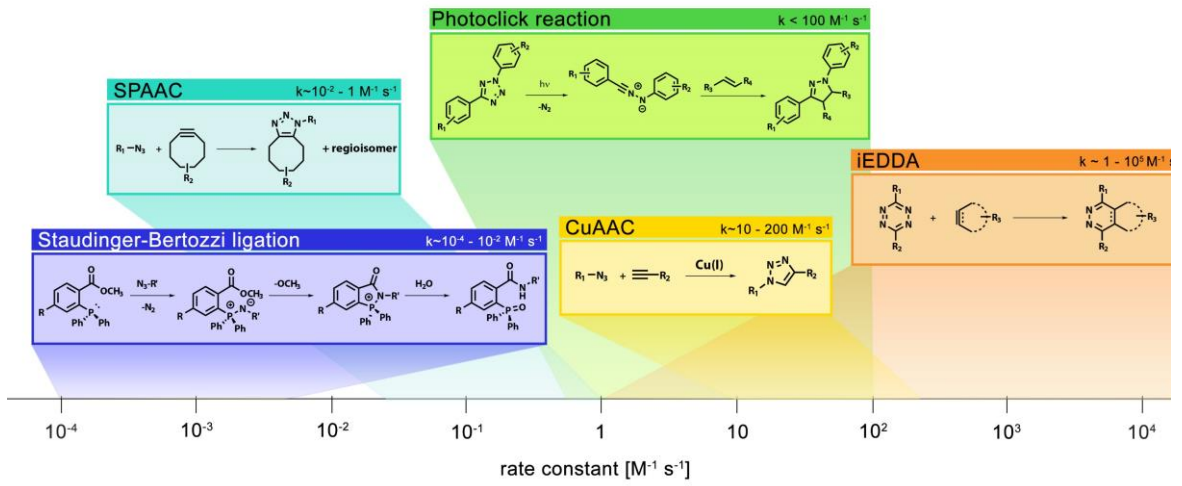

Figure 1. Scheme of bioorthogonal reactions with the range of their rate constants

phenyl-1,2,4,5-tetrazine (H-Tet) as the quencher moiety. The major difference between the two tetrazines is their different reactivity and chemical stability. While H-Tet reacts approximately 30-times faster in click reactions it exhibits a lower chemical stability under physiological conditions.[18] It should be noted that the last decade has brought the development of further bioorthogonal ligation reactions[19,20,21] yet it is the inverse electron demand Diels-Alder reaction (iEDDA) of tetrazines and strained alkenes/alkynes that is still on the rise in terms of the applied bioorthogonal ligation step. It should be pointed out, however, that the easy metabolic incorporation of azide appended chemical reporters keeps SPAAC based tagging schemes in the race.[4]

Finally, it is important to mention that the role of bioorthogonal chemistry has been recently expanded to bond cleavage reactions.[22] These reactions include a bioorthogonal click step that induces a cascade of subsequent steps leading to elimination of a substituent from a TCO (click-to-release TCO, crTCO)[23,24] or a tetrazine scaffold (click-to-release tetrazines, crTZ)[25]. Such bioorthogonal cleavage reactions are already the subject of clinical trials for advanced solid tumors and non-Hodgkin lymphoma.[26,27]

Mutually orthogonal bioorthogonal reactions

Not only are bioorthogonal reactions orthogonal to naturally occurring reactions, but some are also mutually exclusive to each other as well. Such mutually orthogonal bioorthogonal reactions involve pairs of reactions that take place simultaneously and exhibit high chemoselectivity to ensure minimal-to-no cross reactivity.[28] For example, dibenzocyclooctynes are inert to tetrazines due to the presence of an $A^{1,3}$-strain that develops in the transition state, while azides do not react with

trans-cyclooctenes. This allows to conduct highly chemoselective transformation in the collective presence of azides, dibenzocyclooctynes, tetrazines and TCOs.[29] In some instances, large differences in reaction rates also lead to chemoselective reactions, however, in this case sequential administration of one of the reactants is necessary.[30,31] Such mutually exclusive bioorthogonal transformations are quite useful e.g., in multicolor labeling schemes.

Introduction of bioorthogonality to biomolecules

In general, *in cellulo* and *in vivo* bioorthogonal modulation reactions follow a two-step scheme in which the biomolecule of interest is first modified with a chemical reporter - a building block, bearing a bioorthogonal function by means of metabolic incorporation, protein engineering or enzymatic action.[32] The as-bioorthogonalized systems are then targeted selectively with a probe or affinity tag, specific function etc. harboring a complementary bioorthogonal function. Such modulated biomolecules are subsequently used to interrogate biological systems in order to get deeper insight e.g., into biomolecular processes. Bioorthogonal manipulation schemes allow for the modification of virtually any biomolecules. Carbohydrates, lipids and nucleic acids are usually bioorthogonalized by metabolic incorporation of suitable non-canonical metabolites. Oligonucleotides can also be rendered bioorthogonal by means of PCR or solid phase synthesis.[33] Proteins on the other hand are very efficiently modulated with bioorthogonal functions by advanced synthetic biology approaches, such as fusion tags in combination with bioorthogonalized substrates or through site-specific incorporation of non-canonical amino acids (ncAAs) *via* genetic code expansion. While there are examples for the selective labeling of various biopolymers (e.g., carbohydrates,[34,35,36] nucleic acids,[37,38] lipids[39,40] proteins[41,42]) or small molecular ligands[43,44] protein-related bioorthogonal labeling schemes are, by far, the most popular; possibly due to the advanced technology for the incorporation of bioorthogonal handles into proteins.[45] Alternatively, bioorthogonal tags can be introduced *via* ligand directed post-translational or enzyme mediated ligation means.[46,47,48,49]

Bioorthogonalized photoresponsive materials

Covalent modification of biomolecules with various probes and tags has become one of the hallmarks of bioorthogonal chemistry applications. A recent statistical analysis of publication data between 2010 and 2020 revealed that papers demonstrating the robustness of bioorthogonal chemistry aided labeling and imaging of biomolecules in live organisms contribute with the highest percentage to reports in the field of bioorthogonal chemistry applications.[4] Efforts in the past decades have resulted in substantial hardware developments in fluorescence microscopy, which revolutionized the field of optical imaging.[50] The emerged deterministic and stochastic super-

resolution microscopy (SRM) methods allow visualization of cellular structures in their native context at resolutions never seen before. Optical imaging aided interrogation of live systems facilitate the better understanding of cellular processes. In order to take full advantage of the latest hardware developments to be able to study more complex questions the growing demands of the emerged *super resolution microscopy* (SRM) methods need to be addressed.[51] These needs governed recent research trends in the field of fluorescence probes as it is rather the lack of suitable markers that can be considered as the major limitation in fluorescence imaging. Even in case of probes with ideal photophysical characteristics, such as high molar extinction coefficient, fluorescence quantum yield and photostability, the achievable signal-to-noise ratio is often impaired by fluorescence of endogenous fluorophores e.g., NADH, flavins, porphyrins, aromatic amino acids etc.[52] These *autofluorescence*-related issues are efficiently addressed by selecting dyes with excitation bands in the red, far-red or near infra-red region. Alternatively, scaffolds with large Stokes-shifts can also be applied as excitation and emission maxima of natural fluorophores are very close to each other. Synthetic probes can also display considerable background fluorescence, which may compromise the sensitivity and resolution. To address this problem, the use of *fluorogenic probes* is suggested.[53,54,55] Characteristics of fluorogenic probes is that they exist in a quenched state until they are transformed to an emissive form in response to a specific ligation reaction. Further fundamental challenge for probes is posed by the need for selective and site-specific modification of target structures, while keeping the size of the probe as small as possible to minimize functional perturbation and reduce linkage errors, i.e., displacement of the fluorophore from the epitope or tagging site of the target protein.[51,56] Effectively addressing the ultimate challenge of selective and site-specific manipulation of biomolecules, bioorthogonal reactions have become indispensable tools to install small organic, minimally perturbing markers onto various cellular targets. Further to this, a special set of fluorogenic probes has emerged whose fluorescence is turned on in response to a bioorthogonal reaction.[54] There are several bioorthogonal functions that can render probes fluorogenic. These examples involve tetrazoles,[57,58] cyclooctynes,[59,60] linear alkynes,[61] sydnones[62] and most notably azides[36,63] and tetrazines.[64,65]

Further to the installation of probes, activation of photoresponsivity of singlet oxygen sensitizers,[66] photothermal transduction agents[67] or photolabile protecting groups by a bioorthogonal ligation is also possible and represents a very young, emerging research field. The extra level of on-target selectivity and improved spatial/temporal control considerably intensified related bioorthogonally assisted phototherapy research.[68] Bioorthogonal reaction aided photodynamic, photothermal or photoactivated chemotherapy is foreseen to have profound implications in the treatment of various indications.

STRUCTURE OF THE THESIS

In line with the requirements set by the Doctoral Council of the Hungarian Academy of Sciences for the process of applying for the title Doctor of the Hungarian Academy of Sciences, this short thesis is comprised of 20 selected publications, with the corresponding authorship of Péter Kele. Present thesis *conceptually summarizes* the key achievements of the author since 2012 when he established his own research group. These results were mainly achieved in the "Lendület" Chemical Biology Research Group led by the applicant. Within the last 10 years the group has gained considerable, internationally recognized expertise in the design of switchable optical systems and the design of strategies suitable to address complex biological challenges. The group of Péter Kele demonstrated their unique position in the field by pioneering several concepts. For example, they were the first ones to apply mutually orthogonal bioorthogonal reactions. Also, they introduced the concept of multiple fluorogenicity and conditional activatability of photolabile protecting groups to the scientific community.

The *20 publications* (references in red, attached to this short thesis) are grouped into 3 chapters. The content of these chapters are based on the development of the respective bioorthogonal tools with Chemical Biology applications thereof. Major part of this work, Chapter 1 gives insights into the world of bioorthogonally aided fluorogenic probes suitable for optical interrogation of living systems at molecular resolution. Chapter 2 discusses our contribution to the development and understanding of certain bioorthogonal functions that allow manipulation of biomolecules with molecular precision. Chapter 3 summarizes results of bioorthogonally activatable photolabile protecting groups. Although these chapters are seemingly different they all facilitate the better understanding of molecular tools that either help us understanding biological systems or enable us to target diseases with drugs with increased precison. At the end of each chapter valorization and future prospectives are discussed.

Chapter 1 Towards advanced bioorthogonally controlled fluorogenic probes	Reference
Herner, A.; Nikić, I.; Kállay, M.; Lemke, E. A.; Kele*, P., **A new family of bioorthogonally applicable fluorogenic labels**, *Org. Biomol. Chem.* **2013**, *11*, 3297	73
Herner, A.; Girona, G. E.; Nikić, I.; Kállay, M.; Lemke, E. A.; Kele*, P., **New Generation of Bioorthogonally Applicable Fluorogenic Dyes with Visible Excitations and Large Stokes Shifts**, *Bioconjugate Chem.* **2014**, *25*(7), 1370	74
Demeter, O.; Fodor, E. A.; Kállay, M.; Mező, G.; Németh, K.; Szabó, P. T.; Kele*, P., **A Double-Clicking Bis-Azide Fluorogenic Dye for Bioorthogonal Self-Labeling Peptide Tags**, *Chem. Eur. J.* **2016**, *22*, 6382	75
Demeter, O.; Kormos, A.; Koehler, C.; Mező, G.; Németh, K.; Kozma, E.; Takacs, L.; Lemke, E. A.; Kele*, P., **Bisazide Cyanine Dyes as Fluorogenic Probes for Bis-cyclooctynylated Peptide Tags and as Fluorogenic Crosslinkers of Cyclooctynylated Proteins**, *Bioconjugate Chem.* **2017**, *28*(5), 1552	76

Kormos, A.; Koehler, C.; Fodor, A. E.; Rutkai, Zs. R.; Martin, M. E.; Mező, G.; Lemke, E. A.; Kele*, P., **Bistetrazine-cyanines as double-clicking fluorogenic two-point binder or crosslinker probes**, *Chem. Eur. J.* **2018**, *24*, 8841	77
Kozma, E.; Estrada Girona, G.; Paci, G.; Lemke, E. A.; Kele*, P., **Bioorthogonal Double-Fluorogenic Siliconrhodamine Probes for Intracellular Superresolution Microscopy**, *Chem. Commun.* **2017**, *53*, 6696	78
Kormos, A.; Kern, D.; Egyed, A.; Söveges, B.; Németh, K.; Kele*, P., **Microscope laser assisted photooxidative activation of bioorthogonal ClickOx probes**, *Chem. Commun.* **2020**, *56*, 5425	85
Kormos, A.; Egyed, A.; Olvany, J. M.; Szatmári, Á.; Biró, A.; Csorba, Zs.; Kele*, P.; Németh, K., **A bioorthogonal double fluorogenic probe to visualize protein–DNA interaction**, *Chemosensors* **2022**, *10*, 37	86
Knorr, G.; Kozma, E.; Herner, A.; Lemke, E. A.; Kele, P., **New, red-emitting tetrazine-phenoxazine fluorogenic labels for live-cell intracellular bioorthogonal labeling schemes**, *Chem. Eur. J.* **2016**, *22*, 8972	87
Knorr, G.; Kozma, E.; Schaart, J. M.; Németh, K.; Török, Gy.; Kele*, P., **Bioorthogonally applicable fluorogenic cyanine-tetrazines for no-wash super-resolution imaging**, *Bioconjugate Chem.* **2018**, *29*, 1312	88
Albitz, E.; Németh, K.; Knorr, G.; Kele*, P., **Evaluation of bioorthogonally applicable tetrazine Cy3-probes for fluorogenic labeling schemes**, *Org. Biomol. Chem.* **2023**, *21*, 7358	89
Albitz, E.; Kern, D.; Kormos, A.; Bojtár, M.; Török, Gy.; Biró, A.; Szatmári, Á.; Németh, K.; Kele*, P., **Bioorthogonal Ligation-Activated Fluorogenic FRET Dyads**, *Angew. Chem. Int. Ed.* **2022**, *61*, e202111855	90

Chapter 2 Development and understanding of bioorthogonal tools	Reference
Varga, B. R.; Kállay, M.; Hegyi, K.; Béni, Sz.; Kele*, P., **A non-fluorinated monobenzocyclooctyne for rapid copper-free click reactions**, *Chem. Eur. J.* **2012**, *18*, 822	101
Kozma, E.; Nikić, I.; Varga, B. R.; Aramburu, I. V.; Kang, J. H.; Fackler, O. T.; Lemke, E. A.; Kele*, P., **Hydrophilic trans-Cyclooctenylated Non-Canonical Amino Acids for Fast Intracellular Protein Labeling**, *ChemBioChem* **2016**, *17*, 1518	111
Cserép, G. B.; Demeter, O.; Bätzner, E.; Kállay, M.; Wagenknecht*, H-A.; Kele*, P., **Synthesis and Evaluation of Nicotinic Acid Derived Tetrazines for Bioorthogonal Labeling**, *Synthesis* **2015**, *47*, 2738	112
Cserép, G. B.; Németh, K.; Szatmári, Á.; Horváth, F.; Imre, T.; Németh, K.; Kele*, P., **Beyond the bioorthogonal inverse electron demand Diels–Alder reactions of tetrazines: 2-pyrone-functionalized fluorogenic probes**, *Synthesis* **2022**, *54*, A	115
Szatmári, Á.; Cserép, G. B.; Molnár, T. Á.; Söveges, B.; Biró, A.; Várady, G.; Szabó, E.; Németh, K.; Kele, P., **A Genetically Encoded Isonitrile Lysine for Orthogonal Bioorthogonal Labeling Schemes**, *Molecules* **2021**, *26*, 4988	121

Chapter 3 Conditionally activatable photolabile protecting groups	Reference
Bojtár, M.; Kormos, A.; Kis-Petik, K.; Kellermayer, M.; Kele*, P., **Green-Light Activatable, Water-Soluble Red-Shifted Coumarin Photocages**, *Org. Lett.* **2019**, **21**, 9410	142
Németh, E.; Knorr, G.; Németh, K.; Kele*, P., **A Bioorthogonally Applicable, Fluorogenic, Large Stokes-Shift Probe for Intracellular Super-Resolution Imaging of Proteins**, *Biomolecules* **2020**, *20*, 397	146
Bojtár, M.; Németh, K.; Domahidy, F.; Knorr, G.; Verkman, A.; Kállay, M.; Kele*, P., **Conditionally activatable visible-light photocages**, *J. Am. Chem. Soc.* **2020**, *142*, 15164	147

1. TOWARD ADVANCED BIOORTHOGONALLY CONTROLLED FLUOROGENIC PROBES

Selective and site-specific installation of markers onto biomolecules is still the major application of bioorthogonal chemistry aided chemical biology approaches. Due to latest hardware developments in fluorescence microscopy, it is rather the lack of suitable probes that is considered as major limitation in the field of fluorescent microscopy imaging.[56] In order to take full advantage of the emerged super-resolution methods and to address more complex questions novel probes are needed to address the growing demands of super resolution microscopy (SRM) methods. These new demands dictate that besides favorable optical features such as brightness, red-excitability, or large Stokes-shifts, fluorogenicity and photostability, novel probes should also address the challenge of specific installation, while keeping the level of perturbation as low as possible.

A. Challenges

Bioorthogonally applicable markers allow the use of minimal tags harboring a complementary bioorthogonal function. Such labeling schemes enable installation of fluorescent probes with minimal structural and functional perturbation of the protein of interest (POI) while keeping linkage error as low as possible. Ideal fluorescent markers possess high molar extinction coefficient and fluorescence quantum yield to ensure acceptable brightness. At the same time, these probes need to feature acceptable photo- and biological stability. Since chemical biology related research topics focus on the study of live systems, further requirements are set in order to allow fast (no-wash) imaging with high signal-to-noise ratio. With the careful selection of the fluorescent core autofluorescence is easily suppressed. Keeping background fluorescence low, however, demands special solutions. Certain bioorthogonal motifs can be applied as two-in-one handles that ensure specific conjugation to targets and render selected fluorescent cores fluorogenic at the same time. Salient feature of these bioorthogonalized fluorogenic scaffolds is that their fluorescence is quenched until the bioorthogonal unit is transformed in the ligation step. The goodness of fluorogenicity can be characterized by several ways e.g., by the ratio of the fluorescence intensities, quantum yields or brightnesses of the conjugated and unconjugated free forms at a given wavelength. Comparison of brightness is probably the most practical from the imaging point of view as it also encompasses changes in absorptivities upon conjugation. Generally, the larger the increase in the respective term, the higher the achievable contrast is and consequently the less it is required to wash off unreacted probes. Experience suggests that even one order of magnitude difference is enough to tell specifically bound and freely diffusing or non-specifically adsorbed species apart. Indeed, this two-in-one combination of being a bioorthogonal handle and a quencher

of fluorescence was exploited in the design of various fluorogenic scaffolds. These examples highlighted the strengths but also the limitations of bioorthogonally governed fluorescence modulation strategies in terms of ligation schemes and fluorogenic behavior. These suggest that the quenching efficiency of the most robust bioorthogonal quencher moieties i.e., the azide and tetrazine declines dramatically toward the biologically preferred red range of the spectrum. These prompted research directions that aimed at improving the fluorogenicity of azide / tetrazine-modulated red-excitable probes.

B. Discussion of results

Azides and tetrazines can exert their quenching effects through various mechanisms.[69] For example, the emission intensity of various blue/green excitable cores, e.g., coumarins and BODIPYs, is very effectively modulated by energy (e.g., FRET,[70,39] TBET[64,65]) or electron transfer[71,72] processes. On the one hand, quenching *via* FRET demands matching emission and absorption spectra between the fluorophore and the tetrazine. On the other hand, TBET and PET-based quenching requires matching excited states between the two moduls, which explains the diminished quenching efficiency of tetrazines. Our initial attempts to access fluorogenic scaffolds with red-shifted emission involved azide appended frames that possessed large Stokes-shifts. Theoretical studies with these probes revealed a novel, rotation based quenching mechanism for probes where the azide is directly appended to the fluorescent frame.[73] We found that upon the rotation of the azide group the primarily excited first excited state of these probes interchanged with a non-emissive state. Due to the conformational change of the azide group, a state-switch takes place which opens up the possibility of the internal conversion of the two lowest excited states followed by another internal conversion process to the ground state. However, as we applied this quenching mechanism to more red-shifted probes with extended π-conjugation, effects of such rotation-based quenching became less significant.[74] This prompted us to apply two azide moieties attached directly to the fluorescent core at two distant sites.[75] We hypothesized that the restricted conformational freedom of the cyclic click-product formed in a reaction with a bifunctionalized

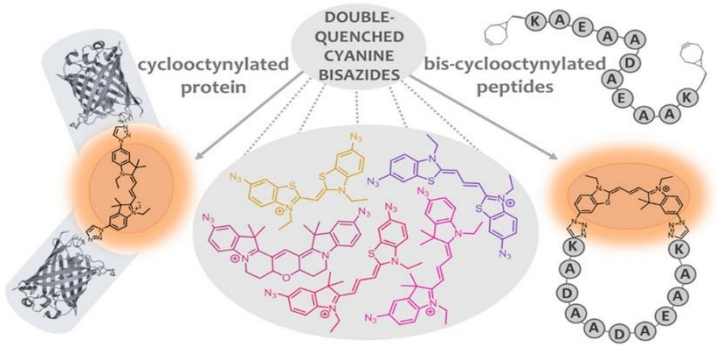

Figure 2. Schematic representation of bistetrazinylated cyanine probes and their conjugation to a bis-functionalized peptide platform or as cross-linkers

target platform will result in increased fluorescence even in case of π-extended scaffolds. We tested our hypothesis on a bis-azido-benzothiazolyl-coumarin framework and mapped its fluorogenic performance against various biscyclooctynylated peptide sequences to find the optimal length of the target platform. Theoretical calculations underlined the importance of the conformation of the cyclic conjugates as the fluorescence of the final products was largely dependent on the dihedral angles between the two aromatic cores of probe. Inspired by these findings we applied the same strategy to various green-yellow absorbing cyanine cores (Figure 2). Compared to other fluorogenic cyanines, these double-quenched systems showed remarkable fluorescence intensity increase upon formation of cyclic dye–peptide conjugates.[76] We also studied the contribution of the individual azide moieties to the quenching of fluorescence. In combination with an optimized 11-mer biscyclooctynylated peptide sequence, remarkable fluorescence intensity increase values were observed (up to 40-fold) upon formation of cyclic dye–peptide conjugates. Furthermore, we also demonstrated that these bisazides are useful fluorogenic cross-linking platforms that are able to form a covalent linkage between monocyclooctynylated proteins. Later we replaced the azide moiety with tetrazine and obtained bistetrazinylated cyanines that were less prone to photodegradation and allowed faster labeling reactions in protein labeling schemes.[77] These double-quenched, bioorthogonally applicable fluorogenic cyanine probes with emission maxima between 600 and 620 nm and excitation wavelengths match well with light sources of fluorescent microscopes. We have studied the fluorogenic potential of the probes upon reaction with our optimized biscyclooctynylated peptide and observed 10-fold enhancements in fluorescence. We also explored the labeling potential of these probes with a double-tagged protein, where two bioorthogonalized ncAAs were implemented through Amber suppression (GCE) technology. Experiments indicated formation of the expected cyclic probe–protein conjugate. However, the moderate fluorogenicity and the complicated synthetic biology task (double amber suppression of a fusion peptide tag) required to create a suitable target platform did not prove this approach very appealing. Driven by the ultimate goal to access red-absorbing probes with improved bioorthogonally driven fluorogenicity we sought for

alternative solutions. Upon considering the limitations of bioorthogonal moiety based quenching we suggested to combine it with distinct quenching mechanisms rather than double the number of the same quencher moiety. This enables the use of routine single ncAA incorporation schemes as well. The silicorhodamine (SiR) frame is a widely used membrane-permeable NIR dye suitable for SRM applications. Besides the high photostability and brightness, the unique environment-dependent fluorescence of carboxy-SiRs due to a polarity dependent lactone-formation resulting in a leuco form allows the fabrication of polarity-based fluorogenic probes. We intended to combine this valence tautomerism-based structural fluorogenicity with tetrazine driven quenching.[78] To this end we designed and synthesized various SiR-Tet derivatives and tested them for fluorogenic behavior in combination with various dienophiles. To our delight a 20-40-fold increase of fluorescence was observed, which -at that time- was exceptional in this wavelength range. Although originally we proposed that the combined quenching effects lead to improved fluorogenicity, based on recent reports by Urano[79] and Wombacher[80], we recently revised this mechanism of action and suggested that the two processes i.e., the bioorthogonal reaction and the tautomerization are not independent of each other but rather the electronic changes as a result of the iEDDA step shift the equilibrium between the non fluorescent spirolacton form and the fluorescent zwitterionic species toward the fluorescent form (Figure 3).

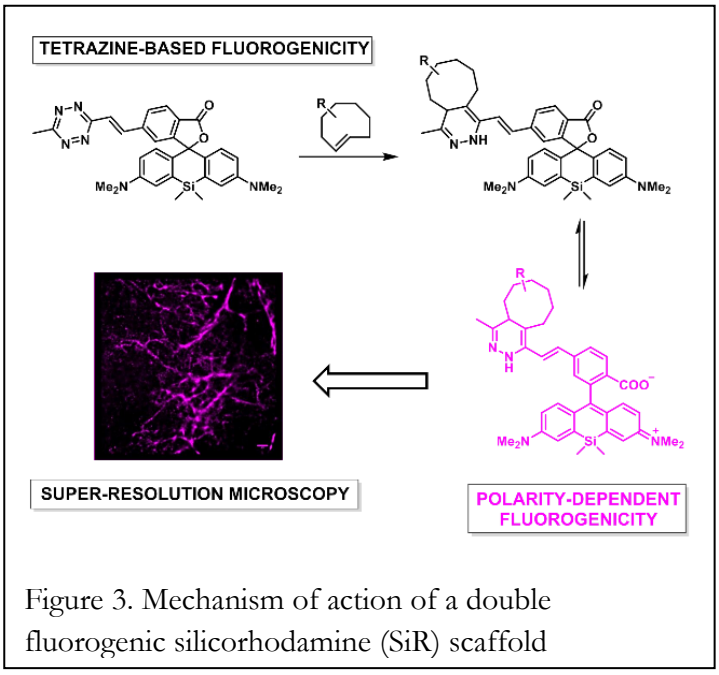

Figure 3. Mechanism of action of a double fluorogenic silicorhodamine (SiR) scaffold

We harvested this iEDDA triggered shift of the lactonization equilibrium in the design of a self-oxidizable fluorogenic probe. Phenothiazines are known for their ability to sensitize $^1O_2$ upon light irradiation.[81,82] The generated reactive oxygen species then oxidize the phenothiazine leading to the formation of sulfoxide or sulfone products, which possess considerably altered spectroscopic features compared to the parent phenothiazine.[83] The phenozhiazine motif is present in rhodafenothiazines that are also capable of undergoing the spirolactone-zwitterionic equilibrium.[84] We proposed that installation of a tetrazine moiety *via* a vinylene linkage will promote a shift in the equilibrium and thus result in substantially altered $^1O_2$ sensitizing ability.[85] Indeed, we have tested the tetrazine and its respective iEDDA product and observed a marked difference (i.e., 20 vs.

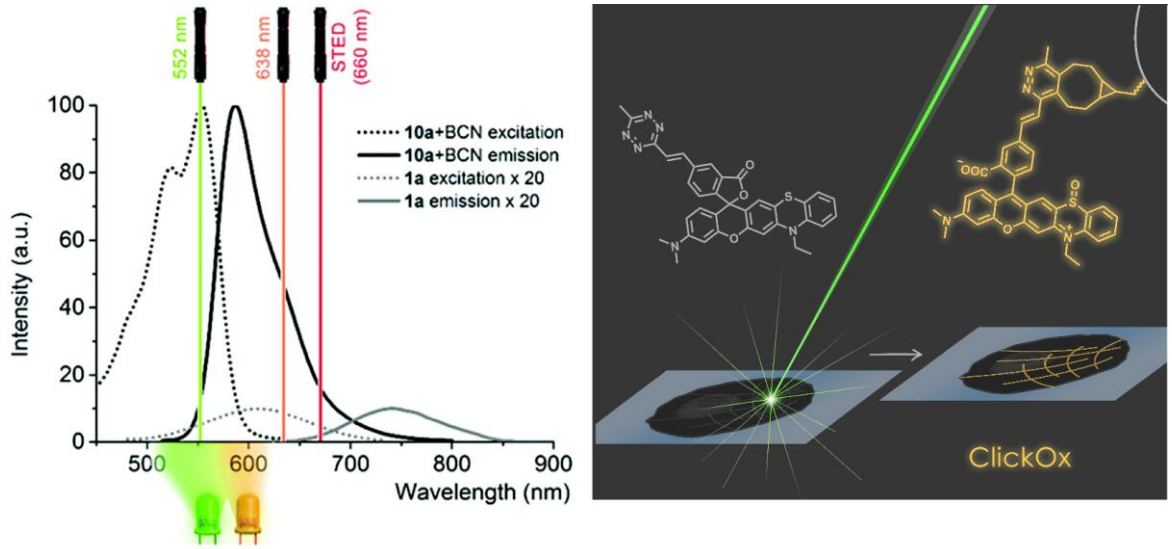

Figure 4. Excitation and emission spectra of ClickOx probe and its BCN conjugate. Schematic represetation of fluorogenic tagging with ClickOx tagging

>90%, respectively) in the conversion of the starting materials to the respective sulfoxides. Furthermore, we observed a pronounced difference in the absorption and emission characteristics of the tetrazine and the iEDDA species. While the tetrazines possessed absorption maximum at around 610 nm with very weak emission intensity at 740 nm, the oxidized iEDDA product showed very intensive absorption and emission at 550 nm and 600 nm, respectively. The overlapping absorbances of the tetrazine and oxidized iEDDA forms allowed us to use the built in 552 nm laser of a confocal microscope to induce oxidation of the iEDDA products and excite fluorescence subsequently (Figure 4). At the same time non-reacted tetrazine probes (dubbed as ClickOx) did not contribute significantly to fluorescence even if oxidized, allowing us to use no-wash labeling schemes. On the top of these, the in situ formed fluorescent form was suitable for STED microscopy as well.

Meanwhile, we recently introduced a double fluorogenic probe suitable to tackle DNA-protein interactions, where the tetrazine-based and structrural fluorogenicity act independently rendering the system an AND logic switch.[86] In these latter examples tetrazine was anchored to various DNA-intercalator scaffolds. The probes were evaluated in terms of double fluorogenic characteristics in the presence/absence of DNA and a complementary bioorthogonal function. Our studies revealed that tetrazine appending thiazole orange derivatives show remarkable double fluorogenic features. One of these probes, a membrane permeable tetrazine modified thiazole orange derivative, (TOT) was further tested in live cell labeling studies. Cells expressing bioorthogonalized DNA-binding proteins showed intensive fluorescence, characteristics of the localization of the proteins upon treatment with our double fluorogenic probe. On the contrary,

labeling similarly bioorthogonalized cytosolic proteins did not result in the appearance of the fluorescence signal (Figure 5).

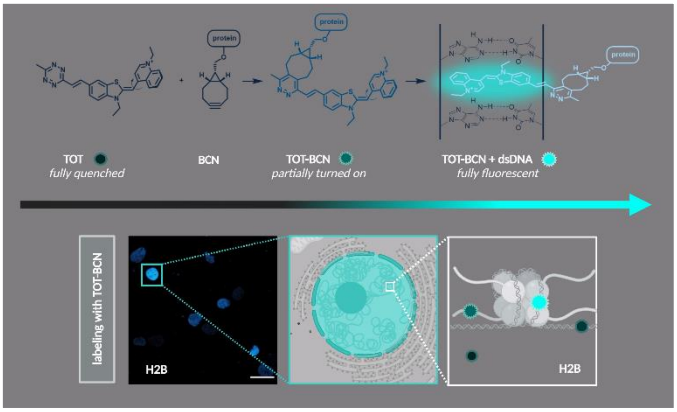

Figure 5. Schematic representation of the mechanism of action for double fluorogenic TOT probe.

Paralell to these, we launched a systematic study that aimed at investigating the mechanism of quenching exerted by the tetrazine. Studies with phenoxazine [87] and cyanine scaffolds [88] linked to a tetrazine motif *via* a phenylene or a vinylene linker suggested that in case of phenylene linkage its restricted rotation leads to electronic decoupling of the two units allowing through-bond energy transfer to be the prevailing mechanism of quenching. In case of vinylene linkage, however, the considerable red-shifts of the absorption and emission maxima indicate a conjugated relationship between the tetrazine and the fluorophore cores, which suggests a different quenching mechanism. Moreover, this latter allowed more efficient quenching compared to TBET. A careful evaluation of tetrazine quenched Cy3 scaffolds involving direct tetrazine attachment or through phenylene or vinylene linkage revealed that in case of direct or vinylene linkage assisted conjugation of the tetrazine to the fluorophore enables an internal conversion based quenching mechanism. [89] Theoretical studies imply that the S_2 (LUMO+1) state is formed predominantly corresponding to a π–π* transition of the chromophores as suggested by the oscillator strengths. The high probability for the LUMO+1 → HOMO transition contradicts the quenched fluorescence, which implies rapid internal conversion to a dark state. This darkstate was verified to be the S_1 (LUMO) state corresponding to the n–π* (HOMO–1 → LUMO) transition of the tetrazine unit. Originally we have described this internal conversion (IC) based quenching for vinylene linked tetrazine-coumarin systems, where it has led to extremely efficient quenching due to the fact that there is a considerable energy difference between the S_2 and S_1 states. In case of cyanines, however, the energy levels of the S_1 and S_2 states are very close to each other allowing reversible transition between the S_1 and S_2 excited states leading to less efficient quenching. On the one hand, these findings explain why conjugated Cy3–tetrazines are more fluorogenic than TBET-governed probes. On the other hand, they also reveal the wavelength limitation of the highly efficient IC-based quenching mechanism.

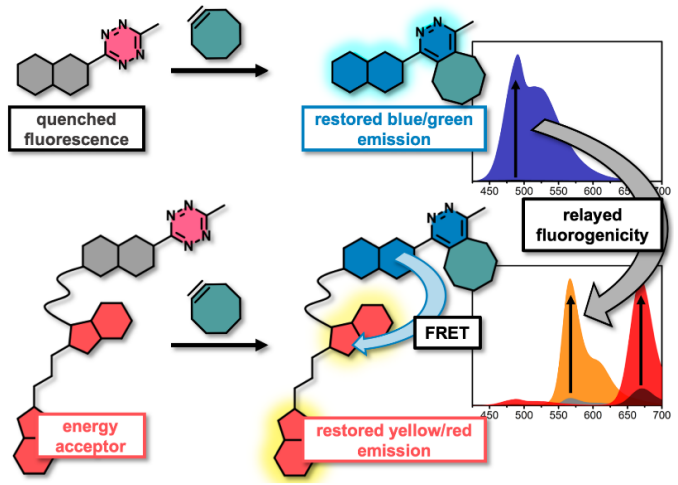

Figure 6. Schematic representation of multichromophoric design and mechanism of action.

Accepting that tetrazines are most efficient in terms of quenching in case of blue – green absorbing/emitting cores, we proposed a conceptually different design for fluorogenic systems based on Förster Energy Transfer. The multichromophoric donor-acceptor systems are composed of an ultrafluorogenic vinyltetrazine appended coumarin and a yellow (Cy3) or red (Cy5) emitting frame. We hypothesized that in such energy transfer dyads the ultrafluorogenic features are relayed to the acceptors by FRET, leading to improved yellow/red fluorogenicities (Figure 6).[90] Indeed, the proposed relay mechanism resulted in improved cyanine fluorogenicities (up to 16-fold for Cy3 dyad) together with increased photostabilities and large apparent Stokes-shifts allowing lower background fluorescence even in no-wash bioorthogonal fluorogenic labeling schemes of intracellular structures in live cells (Figure 7). When compared to the corresponding tetrazine quenched cyanine probes, we could conclude that the dyads allowed much less background signal. Further to this, these multichromophoric systems possessed better overall photophysical features than the individual components. The large apparent Stokes-shift resulting from the non-radiative relay of excitation energy from the donor to the acceptor allowed complete separation of excitation and emission bands eliminating self-quenching or errors during detection due to backscattering. These energy transfer dyads sharing the same donor moiety together with their parent donor molecule allowed three-color imaging of intracellular targets using one single excitation source with separate emission windows. The improved photostabilities[91] allowed prolonged or more intense irradiation, which was harvested in sub-diffraction imaging of intracellular structures using the bioorthogonally activatable FRET dyads by STED microscopy is also presented.

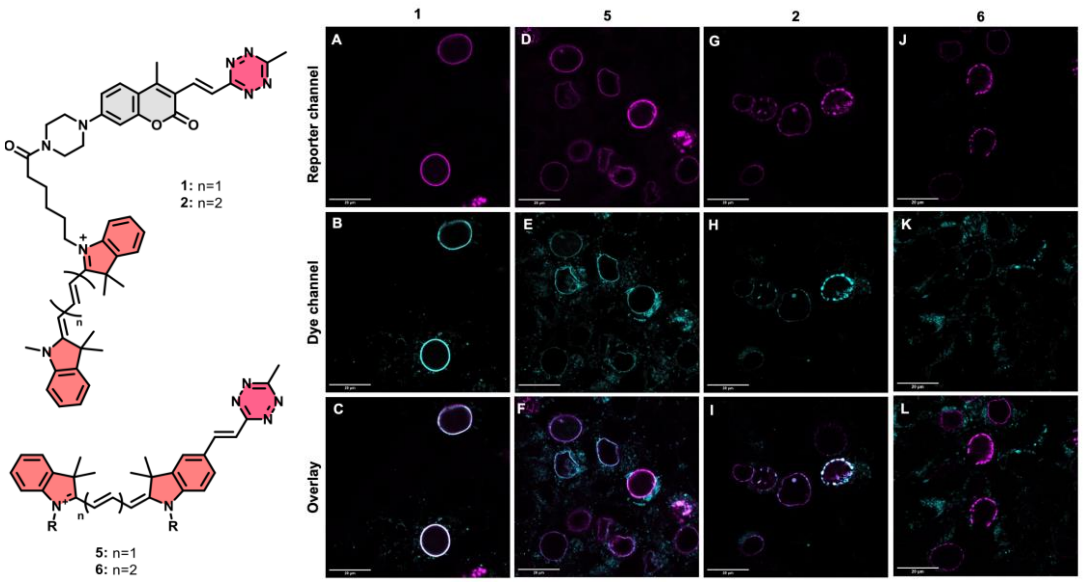

Figure 7. Structure of multichromophoric dyads and their corresponding tetrazine quenched congeners and confocal microscopy images of Lamin-L^{TAG}-miRFP (A–F) or Lamin-GFP^{TAG} (G–L) expressing HEK293T cells treated with Lys(BCN) and probes. Scale bar: 20 μm.

C. Evaluation of results and future directions

The better understanding of the underlying quenching mechanism in bioorthogonally applicable fluorogenic probes is certainly a step forward to develop better probes for super-resolution imaging applications. While live cell technologies are preferred, as specifically targetable fluorogenic platforms can have a large impact on our understanding of biomolecular processes e.g. in order to identify new druggable sites, even those probes that perform "only well" under fixed conditions will depict a great advance. The use of fluorogenic probes exerts an advantage as cumbersome purification procedures can be avoided. Thus, even new dyes, that are seemingly not the "dream" ones, might have a large impact on very important applications such as proteomics, as well. Fluorogenic labelling strategies of purified proteins would permit wash free FRET experiments, which would also depict a major advance over existing approaches like in the understanding of protein-protein interactions.

Each of the above discussed approaches aimed to boost up the fluorogenicity of bioorthogonally applicable red / far red emitting probes has resulted in improved FE values allowing improvement of super-resolution imaging of cellular structures. Yet, the ever-increasing resolution of novel techniques demands even better performance in terms of background suppression. Recent results by Wombacher et al.[72] applies a design concept that allows to push the limits of fluorogenic tetrazine-xanthene probes to unseen FE values. Their red and far-red fluorogenic tetrazine–probes

place the tetrazine and a xanthene core at minimal distance *via* a flexible linker allowing efficient fluorescence quenching *via* Dexter electron exchange mechanism. This design led to a 50-fold far-red fluorogenicity, which is still by far from the excellent FE values of blue excitable probes. On the one hand this suggest that novel design strategies need to be developed that enable even better performance. On the other hand, conceptually different approaches that reconfigure the role of the bioorthogonal motif are also promising directions toward improved bioorthogonally applicable fluorogenic platforms. Our research group is currently exploring two of such conceptually novel designs. One of these is a FRET modulated probe in combination with click-to release chemistry. In these probes a suitable far-red excitable core is linked to a black-hole quencher (e.g., BHQ-3) functionalized crTCO. Upon careful design, iEDDA triggered disassembly of such quenched probes results in covalently attached probes to target structures with restored emission. Another approach may address the challenge by applying a so-called proto-fluorophoric design, where the fluorescent core is formed spontaneously following a bioorthogonal reaction, where again, click-to-release platforms are key elements.

2. DEVELOPMENT AND UNDERSTANDING OF BIOORTHOGONAL TOOLS

The interdisciplinary field of chemical biology aims to study biological systems in their native environment, through small synthetic manipulations by applying chemical tools and analysis. Since the introduction of bioorthogonal reactions, such small molecular manipulation schemes have become more straightforward, more selective, and more biocompatible. Deeper understanding of the mechanism of these reactions facilitates the development of novel functional groups enabling more robust reactions. Getting insight into the scope and limitations of a given bioorthogonal reaction also allows us to develop mutually orthogonal bioorthogonal reaction schemes.

A. Challenges

While bioorthogonal reactions made live biomolecular modifications considerably simpler and more efficient, increasing demands for multiple modulation schemes posed further challenges. These include the development of mutually exclusive chemistries that allow two (or more) bioorthogonal reactions to be carried out in parallel while achieving full chemoselectivity.[28,30,92] Mutually orthogonal bioorthogonal reactions were demonstrated e.g. by the author[93] at an early stage of the bioorthogonal era, however, most of these chemoselective transformations were achieved by sequential administration of reagents to avoid cross-reactivities by full consumption of the possibly interfering functions. Site-specific installation of mutually orthogonal functions is also challenging. While it is possible to combine e.g., genetic code expansion techniques with other labeling strategies, such as self-labeling enzymes or fluorescent proteins it comes at the cost of linkage error. To harvest the full potential of super-resolution techniques to achieve a resolution of proteins that matches size resolution of cryoelectron tomography the use of minimal genetic tags is necessary for the implementation of bioorthogonal tags.[94] Installation of mutually orthogonal bioorthogonal functions onto biomolecules purely by means of GCE is possible, however, it is still not routine although considerable advances were made in this field, including e.g., engineered organelles.[95,96,97,98,99] Further to these, the existing mutually orthogonal bioorthogonal reactions possess substantially different reaction kinetics and laborious optimization process is required in terms of labeling concentration, time or administration order. Also, with rare exceptions these allow only dual color labeling of biological targets. Therefore, novel mutually orthogonal bioorthogonal reactions and bioorthogonalization strategies that address these limitations need to be explored. Wider application of site-specific bioorthogonal labeling of proteins is further hindered by technical limitations. For example, it is challenging to effectively remove unincorporated chemical reporters from live cells. Since this can compromise the efficiency

of the best-performing fluorogenic probe, development of more hydrophilic bioorthogonally applicable ncAAs is needed that can be implemented *via* GCE approaches.

B. Discussion of results

Cyclooctynes are versatile bioorthogonal platforms as they can participate in SPAAC and iEDDA reactions depending on the reaction partner. The robustness of GCE for the site-specific incorporation of bioorthogonal functions calls for the development of synthetically accessible, reactive, and compact motifs. At the same time for the above-mentioned reasons the hydrophobic character of such hydrocarbon platforms needs to be reduced as much as possible. Reported dibenzocyclooctynes are indeed derivatives with increased reactivity, however, the two benzene rings fused to the cyclooctyne considerably increase the hydrophobic character as well. Trying to keep the balance between activity, stability and hydrophilicity, our interest turned to monobenzocyclooctynes. Following the theoretical lead by Goddard et al.[100] we focused on monobenzocyclooctynes that involved an aryl fusion at the 5-6 positions of the cycloctyne. It is noteworthy that these monobenzocyclooctynes have predicted ring strains comparable to that of dibenzocyclooctynes, however with lowered $A^{1,3}$ strain during the cycloaddition process, a problem that compromises the reactivity of dibenzocycloctynes when reacting with azides. Moreover, the considerable $A^{1,3}$ strain present in dibenzocyclooctynes prevents their reaction with tetrazines in iEDDA reactions. In order to experimentally investigate the reaction kinetics of such a monobenzocyclooctyne we have designed carboxymethylmonobenzocyclooctyne, COMBO (2-methoxycarbonyl-5,6,9,10-tetrahydro-7,8-didehydrobenzo[8]annulene), a molecule lacking fluorine substituents and carrying a carboxylic function that allows for further modification.[101] In reference to cyclooctynes that contain aryl groups fused at the 5,6-positions, Krebs et al.[102] and Meier et al.[103] noted that these regioisomers were thermally unstable and only the somewhat more stable 3,4-benzannulated regioisomers could be accessed in poor yields. We hypothesized that the reason for their results was the harsh conditions (e.g., 190 °C) applied when forming the alkyne moiety. Therefore, we sought for chemical transformations that allowed mild conditions without the need for expensive and sensitive reagents. Following several unsuccessful attempts, we devised a synthetic route that included an iEDDA reaction as a key step (highlighted in

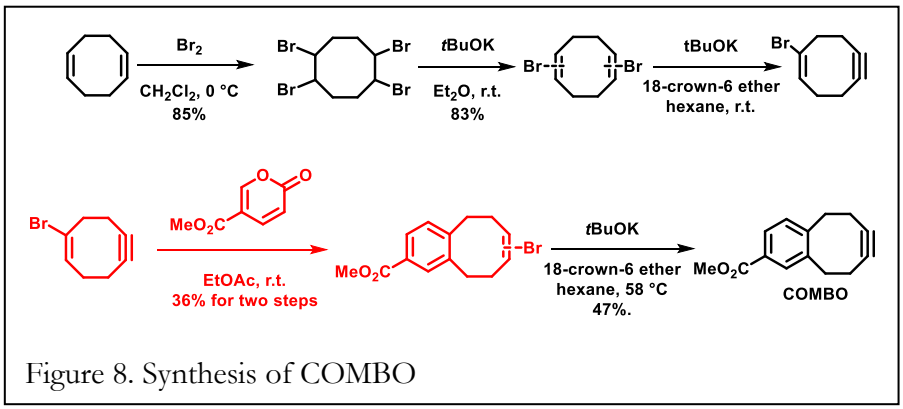

Figure 8. Synthesis of COMBO

red in Figure 8). To our delight, we have found the benzocyclooctyne stable and under the mild conditions applied here it is formed with a reasonable yield. Hydrolysis of the methyl ester under strongly basic conditions at 30 °C for 2 h, followed by acidic work up gave COMBO-acid in 92% yield indicating that the benzocyclooctyne moiety is stable under these conditions. Stability of COMBO was further assessed under various conditions e.g., at 37 °C in aqueous solution or in the presence of gluthathione and gave satisfactory results in all cases. We have also elaborated the reactivity of COMBO. Reaction with benzyl azide indicated the sole formation of products (ca. 1:1 mixture of regioisomers). The gratifying results have shown a second order rate constant of k_2 = 0.235 ± 0.006 $M^{-1}s^{-1}$ in acetonitrile and k_2 = 0.795 ± 0.007 $M^{-1}s^{-1}$ in water-acetonitrile 3:4 v/v. This reactivity is in the same range with dibenzo fused congeners, however, COMBO has a much lower lipophilicity (i.e., ᶜlogP = 1.9). Later, we have demonstrated the versatility of the COMBO motif in several studies involving COMBO modified fluorescent probes,[101, 104 , 105] cross-linkers [106] or nucleotides[107] (these papers are not part of the thesis). We have tried to encode Lys(ε-*N*-COMBO) into proteins by means of GCE using mutant PylRS/$tRNA^{Pyl}$ pairs, however, these attempts failed. This and the mass production of somewhat less reactive BCN (bicyclo[6.1.0]nonyne) and its derivatives marginalized the significance of COMBO.[108, 109]

In a collaborative project with Harvard Medical School, we synthesized a set of hydrophilic TCO derivatives that included exo- or endocyclic heteroatoms.[110] These TCOs showed good to excellent reactivities thus we wished to investigate their potential as parts of ncAAs in GCE, for the rapid labeling of intracellular proteins in mammalian cells. Also, we aimed at providing experimental evidence for the enhanced clearance characteristics of unincorporated ncAAs from cells. To this end, we modified lysine at its *ε*-amino function with various TCO scaffolds *via* a carbamate linkage.[111] In order to evaluate the success of genetic encoding of these ncAAs, green fluorescent protein with an amber mutation at position 39 (GFP^{Y39TAG}) was used. We co-expressed wild-type and previously reported[42] mutant (PylRS(Y306A, Y384F)^{AF}) PylRS/$tRNA^{Pyl}$ pairs from *M. mazei* in the presence of ncAAs in *E. coli* and mammalian HEK293T cells. In-gel and confocal microscopy analyses showed that a dioxolane derived TCO (DOTCO) appended Lys (Lys(ε-*N*-DOTCO) is efficiently translated by the translation machinery of cells. Further studies with DOTCO-Lys tagged, GFP

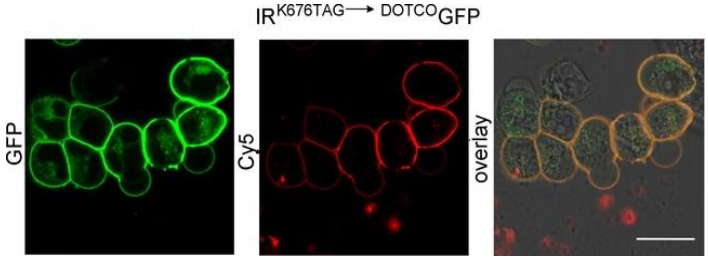

Figure 9. Cell-surface labeling of insulin receptors by the genetic encoding of DOTCO-Lys. $IR^{K676TAG→DOTCO}$GFP expressing HEK293T cells were labeled with Cy5-H-Tet. Scale bar: 20 mm

fused insulin receptors ($IR^{K676TAG \rightarrow DOTCO}GFP$) expressing cells in combination with a tetrazine modified fluorescent probe (Cy5-H-Tet) resulted in specific red fluorescent labeling of the cell-surface IRs (Figure 9). To assess clearance characteristics of unincorporated ncAAs from cells, we performed a comparative washout assay. For comparison we involved commercially available ncAAs i.e., Lys(ε-N-BCN) and Lys(ε-N-TCO*) that are routinely used in GCE. During these studies, cell culture medium containing excess ncAA was removed at different time points from nontransfected COS7 cells and residual cytoplasmic ncAA was detected in live cells after labeling with cell-permeable tetrazinylated tetramethylrhodamine probe (5-TAMRA-H-Tet). Our studies indicated that efficient removal of excess Lys(ε-N-TCO*) (clogP = -0.053) from the cytoplasm was not possible, even after 6 h of washing. However, Lys(ε-N-BCN) (clogP = -0.753) gave almost background-free labeling after 6 h, in line with its almost one order of magnitude lower clogP value. Lys(ε-N-DOTCO) (clogP = -2.917) on the other hand, was easily removed from the cytoplasm of cells even after 5 min washing. Further comparative studies with benchmark Lys(ε-N-BCN) revealed that labeling with Lys(ε-N-DOTCO) also allows for higher signal to noise ratio. The remarkable washout rate of Lys(ε-N-DOTCO) is therefore not only advantageous from a practical perspective, but it might also enable studies of proteins with rapid turnover (e.g., many membrane receptors, nuclear proteins or MAP kinases).

We have also investigated the structure-activity/stability relationship of some novel, nicotinic acid derived tetrazines. In a thorough study we aimed at elaborating the role of the steric demand of substituents in the Diels-Alder reactivity of electron deficient tetrazines.[112] We have investigated the reaction rates and physiological stability of model tetrazines in the presence of various dienophiles, like sterically less demanding non-strained ethyl vinyl ether (EVE), less (CyO) and more strained, thus more reactive (COMBO-acid) cyclooctynes and a highly strained cyclooctene (TCO) (Figure 10). Experimental findings indicated that the reactivity order that could have been expected from the LUMO energies of the tetrazines have rather reversed in favor of lower

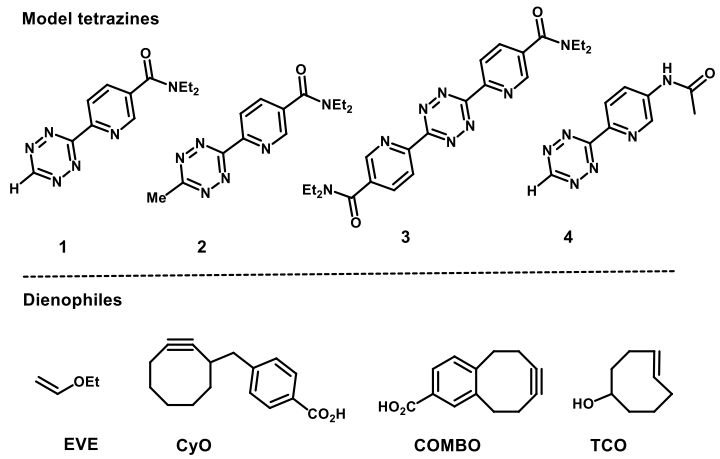

Figure 10. Structure of the studied tetrazines and dienophiles

Table 1 Second order rate constants of the reaction between tetrazines and various dienophiles. The LUMO energies were calculated at the Hartree–Fock level of theory with the 6-311++G** basis set at B3LYP/6-311++G** geometries

tetrazine	$t_{1/2}$	LUMO	k_2 / $M^{-1}s^{-1}$			
	(h)	(eV)	EVE[a]	CyO[a]	COMBO[b]	TCO[b]
1	4	0.71	0.024	6.25	n.d.	n.d.
2	88	0.83	0.00145	n.d.	210	4000
3	4	0.46	0.008	0.23	n.d.	n.d.
4	6	0.69	0.010	2.89	n.d.	n.d.

[a] measured in DMSO/water (9/1 v/v) at 25 °C [b] measured in 1% DMSO in PBS buffer (pH 7.2) at 36 °C

steric demand (Table 1. c.f. compounds 1 and 3). This significance of the steric demand was even more prominent when a sterically more demanding dienophile (CyO) was applied. Theoretical calculations showed good correlations with the experimental results as the free energies of activation were higher for compound 3 with both dienophiles. In addition, the difference between the activation barriers for the reactions of 1 and 3 is somewhat bigger for CyO. Besides reactivity, the stability of these new tetrazines was also studied and it was found that only the least reactive, non-symmetrically decorated tetrazine (2) can stand physiological conditions for longer times. Though this platform showed low second order rate constant with less reactive dienophiles, it provided good rates with more reactive COMBO and TCO. We therefore applied this scaffold in the design of a bioorthogonalized nucleic acid building block. Incorporation of this building block into a DNA sequence using standard solid phase nucleic acid synthesis, a fully automated protocol together with subsequent on-bead labeling experiment revealed that this tetrazine is suitable for the implementation of a reactive bioorthogonal handle into oligonucleotides. As seen, the field of bioorthogonal chemistry and imaging probes have benefited greatly from tetrazines, however, the need for mutually orthogonal bioorthogonal reactions, for example in multi-color labeling schemes, call for the development of alternative bioorthogonal reactions. This includes development of novel dienes for iEDDA schemes with substantially different reactivities toward strained alkenes/alkynes. Such needs were addressed recently by the development of triazines[33] and triazinium salts[113] that react with strained dienophiles in iEDDA reactions. Sydnones can also react with strained alkynes in thermal [3 + 2] cycloadditions.[114] These are very important additions to the bioorthogonal toolbox, offering more options to develop mutually orthogonal bioorthogonal chemistries. While iEDDA reactions of 2-pyrones have been known for quite a while and even we used such platform in the key step toward accessing COMBO, the use of 2-pyrones as bioorthogonally applicable dienes remained unexplored. In line with our involvement in the design and synthesis of bioorthogonally applicable fluorogenic probes for protein labeling purposes and in the context of bioorthogonal toolbox expansion, the 2-pyrone moiety seemed a particularly useful platform. We reasoned that 2-pyrones form a benzene ring with suitable alkynes in an

iEDDA–retroDA reaction sequence, which, upon careful design, would directly result in scaffolds with extended conjugation, giving rise to dramatic changes in spectroscopic properties.[115] To verify the viability of our hypothesis regarding the fluorogenic probe design potential of 2-pyrones, we set forth a study aiming at exploring the applicability of the 2-pyrone scaffold from these two aspects. Literature examples describe reactions of 2-pyrones with alkynes that required catalysts, long reaction times or higher temperatures, however, no studies under physiological conditions are reported.[116, 117] Our pilot experiment of the overnight reaction of methyl coumalate and BCN at room temperature resulted in the iEDDA product in 90% isolated yield. Inspired by this finding, we moved onto our ultimate goal and explored the feasibility of the pyrone moiety in the fabrication of bioorthogonally applicable fluorogenic probes. To this end, we designed and synthesized a set of probes (2-6, Figure 11) furnished with a 2-pyrone moiety. The probes were designed in a way that the forming iEDDA product benzene ring becomes directly attached to a conjugated system, giving rise to π-extended structures. For synthetic considerations, 4-hydroxy-6-methyl-pyrone was chosen as a readily available starting material, which offers a conjugation site *via* its hydroxyl group. We believed that cross-coupling reactions of pseudohalogenated pyrones would allow a versatile approach for the synthesis of a wide variety of 2-pyrone derivatized scaffolds. Following synthesis, we tested their reactions with BCN and monitored the changes in their fluorescence spectra. Results showed that all compounds reacted with BCN, however, the change in the fluorescence properties varied considerably, indicating that the position and the nature of the linkage between the 2-pyrone and the fluorescent frame should be carefully considered during the design process. Probe 4 showed significantly redshifted excitation and emission maxima, both of which were slightly blueshifted upon reaction with BCN. Notably, however, reaction of 4 was accompanied by a huge increase in the intensity of fluorescence (over 100-fold), therefore we further studied this probe. First, we

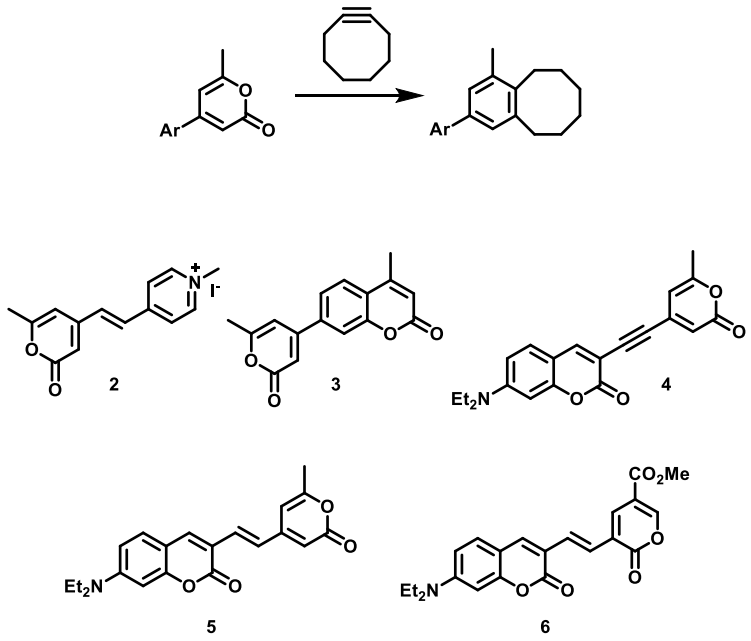

Figure 11. Scheme of the reaction and structure of the studied pyrone appended fluorophores

quantified its reaction speed with BCN and found the second-order rate constant to be k_2 = 0.095 $M^{-1}s^{-1}$, which is in the same order of magnitude as the well-established strain-promoted cycloaddition reactions of azides and strained alkynes (e.g., DIFO and BCN have a k_2 of 0.076 and 0.14 $M^{-1}s^{-1}$, respectively). The stability of compound 4 was assessed in the presence of excess amounts of GSH (1–10 mM). Analysis indicated no substantial change of the pyrone in the presence of up to 5 mM GSH after 24 hours. To test whether the pyrone-derivatized bioorthogonally applicable fluorogenic probe 4 is suitable for protein labeling, we first functionalized a human serum protein, Transferrin (TF, 76 kDa) with BCN. Following incubation and work-up, the samples were subjected to an SDS-polyacrylamide gel, and in-gel fluorescence detection. As expected, fluorescent Transferrin bands occurred only where Transferrin was co-incubated both with NHS-BCN and 4. We also explored the mutual orthogonality of the 2-pyrone moiety in the presence of other bioorthogonal functions. We assumed that, similarly to tetrazines, 2-pyrones are also inert towards sterically demanding dibenzocyclooctyne, DBCO. We have indeed confirmed that probe 4 does not react with DBCO. We then combined excess amounts of DBCO and BCN and added 2-pyrone, bearing probe 4, to this solution. Next, all the remaining BCN was consumed by adding a tetrazine bearing fluorogenic probe, PheCou in excess. Finally, we added an azide-bearing probe, CBRD (both PheCou[146] and CBRD[118] were developed in our group) and the reaction mixture was then analyzed by LC-MS. To our delight, only the three expected products could be detected; namely, 4-BCN, PheCou-BCN, and CBRD-DBCO.

Advanced molecular biology tools such as genetic code expansion based implementation of bioorthogonal handles into target proteins considerably complement and greatly facilitate bioorthogonal chemistry approaches. Although there is a large number of ncAAs that are encodable by means of GCE, there is only a handful derivatives that are used routinely for the site-specific installation of bioorthogonal motifs. The emerging need for such encodable ncAAS and for bioorthogonal functions allowing for mutually exclusive bioorthogonal reactions highlight the importance

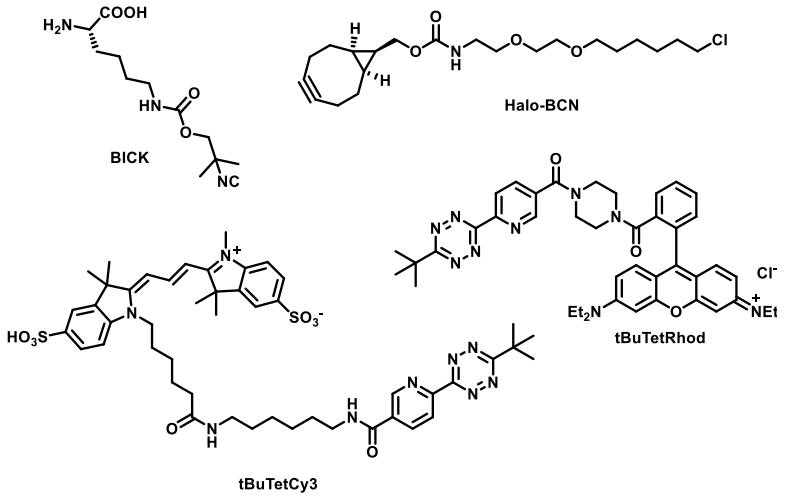

Figure 12. Structure of isonitrile appended ncAA, BICK. Halo-BCN and bulky tetrazine modified fluorescent scaffolds

related research. Wishing to expand the set of genetically encodable ncAAs suitable for mutually exclusive bioorthogonal labeling schemes we turned our attention to the [4 + 1] cycloaddition of sterically hindered isonitriles that are reported to react preferably with bulky tetrazines.[119, 120] We reasoned that the isonitrile group would make an excellent functionality to add to an ncAA, because it is very small and consequently likely to be recognized by one of the previously reported, promiscuous tRNA/tRNA synthetase (RS) pairs like NESPylRS^{AF}/tRNA$^{Pyl}_{CUA}$. Furthermore, the isonitrile function is stable under physiological conditions, non-toxic and very importantly, absent in most eukaryotes. Accordingly, we designed an isonitrile-appending lysine building block, (Lys(ε-N-CH_2-$C(Me)_2$NC), where the isonitrile moiety is linked to the ε-amino function of Lys through a carbamate bond (Figure 12).[121] This bulky isonitrile carbamate lysine, or BICK for short, was then investigated for genetic encodability and the potential for mutually exclusive bioorthogonal reactions. For a complementary function, we have designed a sterically demanding non-symmetrically substituted tBu-tetrazine, (tBuTet), building on our experience with nicotinic acid derivatized tetrazines. The electron withdrawing character of the nicotinic acid motif activates the tetrazine and allows further modification *via* its carboxylic function e.g., with fluorescent probes. Consequently, we synthesized membrane permeable fluorescent probe tBuTetRhod and non-permeable probe tBuTetCy3 (Figure 12). Next, we wished to confirm the different affinities of bulky and non-bulky tetrazines toward our bulky isonitrile and a BCN. To this end, we have combined tBuTet and a sterically allowing tetrazine (Tet), with BICK and BCN. Gratifyingly, LC-MS analysis of the product mixture indicated the formation of only two products corresponding to the adducts of tBuTet + BICK and Tet + BCN, suggesting the potential of these bioorthogonal motifs for mutually exclusive transformations (Figure 13). To further test these mutually orthogonal chemistries in protein labeling studies, we selected two proteins that are matching in size yet can be resolved on SDS-PAGE. Accordingly, we modified Transferrin (TF, 76 kDa) and Bovine Serum Albumin (BSA, (66 kDa) with isonitrile or BCN using NHS-NC or

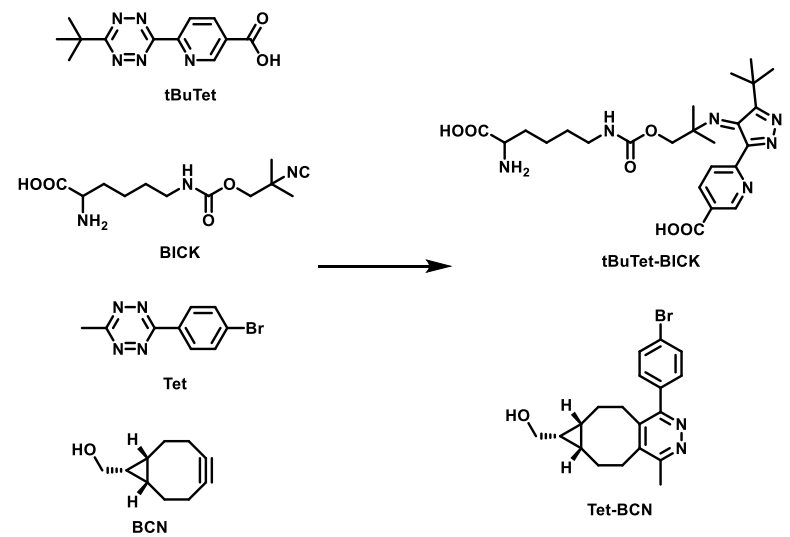

Figure 13. Mutal selectivity of tBuTet and a sterically allowing tetrazine (Tet), with BICK and BCN.

NHS-BCN. The bioorthogonally tagged proteins were then treated with fluorescent probes Tet-SiR and tBuTetRhod either separately or together. The labeled proteins were then subjected to gel separation and imaged (Figure 14). Fluorescent images suggest that sterically allowing Tet-SiR reacted selectively with BCN in either combination.

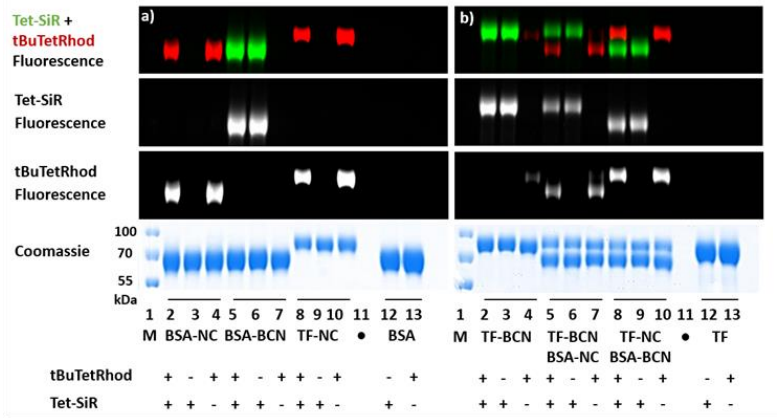

Figure 14. Bioorthogonal labeling of serum proteins Transferrin (TF) and Bovine Serum Albumin (BSA) to test mutual orthogonality between IEDDA and [4 + 1] cycloaddition represented by in-gel fluorescence imaging of SDS-PAGE gels.

Reaction of tBuTetRhod with isonitrile, on the other hand, was only partially selective i.e., no cross reaction was observed with BSA-BCN, while a faint but visible band appeared with TF-BCN. This partial exclusivity was also observed when a mixture of TF-BCN and BSA-NC was labeled with tBuTetRhod alone. When both bioorthogonalized proteins were treated with the two probes, however, the reactions were fully exclusive in either combination, suggesting that partial exclusivity is only present when the BCN moiety is not consumed in the fast iEDDA reaction. The NESPylRS^{AF}/tRNA$^{Pyl}_{CUA}$ pair efficiently translates the amber STOP codon in response to strained alkyne or alkene-containing ncAAs in mammalian cells. Yet, it has been shown to be promiscuous enough to accept further ncAAs.[122] Thus, we tested the ability of NESPylRS^{AF}/tRNA$^{Pyl}_{CUA}$ to incorporate BICK into mammalian proteins. To this end, we transfected HEK293T cells with reporter plasmid mCherry-TAG-EGFP, where TAG represents an Amber STOP codon between the two fluorescent proteins. While red fluorescence helps to evaluate transfection efficiency, appearance of green fluorescence indicates efficient incorporation of BICK. Cells were co-transfected with the NESPylRS^{AF}/tRNA$^{Pyl}_{CUA}$. Microscopy images indicated the appearance of green and red fluorescence in a significant number of cells, suggesting efficient incorporation of BICK (Figure 15). Flow cytometry analysis of cells confirmed this finding as we observed that an average of 81% of transfected cells incorporated BICK. This value suggests similar efficiency to

the incorporation of known substrate Lys(ε-N-BCN), i.e., 76% and significantly higher than the negative control that contains mCherry-TAG-EGFP and NESPylRS^{AF} / tRNA$^{Pyl}_{CUA}$ plasmids, but no ncAA (20%). Given these results we were eager to test mutual orthogonality of the cycloaddition of BICK and the sterically demanding tert-butyl-tetrazine in combination with iEDDA reaction of Lys(ε-N-BCN) and methyl-tetrazine under live-cell conditions. Thus, we incorporated either BICK or Lys(ε-N-BCN) into insulin receptors (IR) bearing an Amber (TAG) mutation at an extracellular position (K676) by transfecting HEK293T cells simultaneously with plasmids coding for the IR^{TAG}GFP fusion construct and the NESPylRS^{AF}/tRNA$^{Pyl}_{CUA}$ pair. Then we carried out single color labeling reactions on both ncAAs with tBuTetCy3 or a sterically allowing TetCy3. Not surprisingly, efficient labeling was seen in case of TetCy3 labeling of Lys(ε-N-BCN), and reassuringly, no Cy3 signal was detected on BICK-bearing cells (Figure 15). Treatment of BICK-expressing cells with tBuTetCy3, however, resulted in robust labeling, although some cross-labeling could be detected as a result of the reaction of tBuTetCy3 with Lys(ε-N-BCN) (Figure 15). Therefore, we reasoned that for dual color labeling schemes, quick saturation of the BCN moieties with sterically non-demanding tetrazine-dyes should be carried out first, followed by the addition of the slowly reacting tBuTet-dye to conjugate to BICK bearing proteins. Next we turned our attention to dual labeling studies in live cells. To this end, we incorporated BICK into the insulin receptor (IR) using the established GCE protocol in HEK293T cells. The second orthogonal bioorthogonal moiety (BCN) was introduced through a plasmid encoding a nuclear protein (LaminA or H2B) fused to the HaloTag self-labeling enzyme. Cells expressing bioorthogonalized IR and HaloTagged LaminA or H2B were then treated with a HaloTag-BCN substrate to introduce the second bioorthogonal

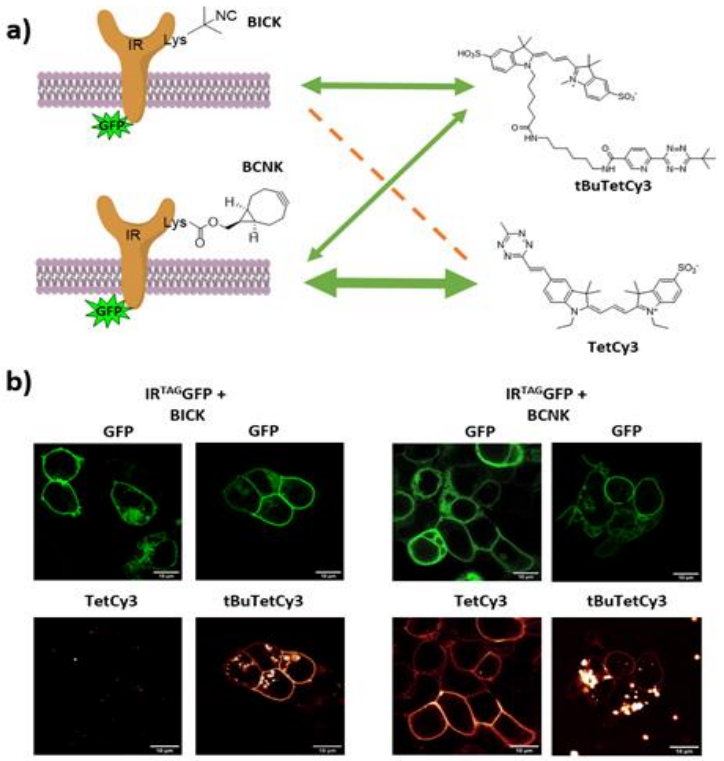

Figure 15. (a) Scheme of insulin receptor-GFP fusion protein (IR^{TAG}GFP) labeling and b) Confocal images of the live cell labeling experiment.

reaction partner to Lamin or H2B. Such double-bioorthogonalized cells were then treated with TetSiR to label the nuclear structures and to consume BCN. Following washing of the cells, the second probe, tBuTetCy3, was applied. Confocal microscopy imaging revealed double bioorthogonal labeling of insulin receptors and histone H2B or LaminA in live HEK293T cells (Figure 16).

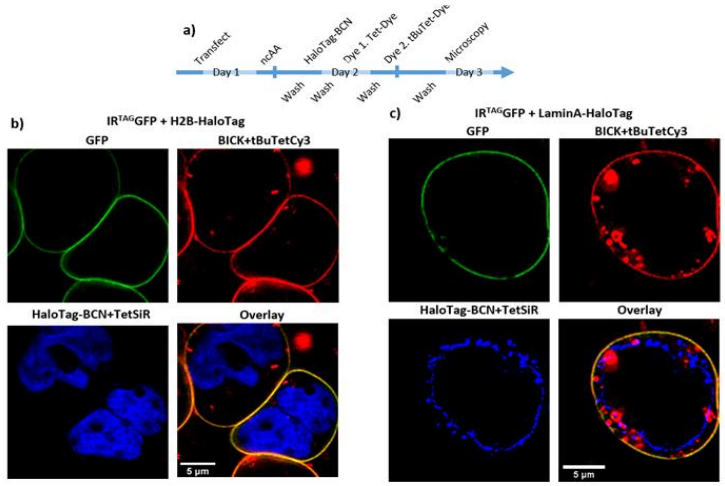

Figure 16. (a) Workflow for live cell dual-color bioorthogonal labeling of overexpressed proteins in HEK293T (b-c) Confocal images of dual-color live cell labeling with the orthogonal-bioorthogonal reactions between BICK and BCN.

C. Evaluation of results and future directions

Multicolor or multimodal labeling schemes place a clear need for mutually orthogonal bioorthogonal chemistries. There is only a handful of such reactions, and these mostly rely on the substantially different reaction kinetics and laborious optimization process is required in terms of labeling concentration, time or administration order. The ideal case would be to have two (or more) such reactions that are truly mutually exclusive and feature similarly high reaction speed. Though the presented results do not offer an ideal combination of reactions, deeper understanding of these chemistries facilitate the development of such reagents and reactions. Along this line, an alternative direction is the use of selectively activatable bioorthogonal functions e.g., by external triggers such as light. In this respect photocaged bioorthogonal functions are foreseen to play an important role. Another aspect to consider is the implementation of bioorthogonal functions to the biomolecules of interest. This suggest development of more and more ncAAs that are suitable for encoding *via* GCE. One future direction is clearly the development of methods suitable for the effective and specific incorporation of more than one bioorthogonal functions possibly allowing mutually exclusive transformations. For this it is important to develop more hydrophilic bioorthogonal platforms that can be easily removed from cells following treatment. Further research directions in the field involve more efficient incorporation of bioorthogonalized building blocks other than amino acids.

3. CONDITIONALLY ACTIVATABLE PHOTOLABILE PROTECTING GROUPS

The noninvasive nature and remote action of light, together with its easy control, fast and cost-efficient operation make related techniques quite appealing. While one of the major application areas in chemical biology is still the selective, site-specific implementation of optically active probes onto various biomolecules for imaging purposes, remarkable developments in the past decade allowed light-related tools to grow from means of observation to a precision tool in chemical biology and medical sciences. Such approaches involve the conversion of the energy of light to chemical energy by means of photoresponsive materials. One group of such photoresponsive compounds is represented by photolabile protecting groups (PPGs or photocages (PCs)), which play an increasing role both in chemical biology studies and in therapeutic applications.

A. Challenges

Recent developments in the field of photoresponsive materials have promoted light-related techniques to a precision tool allowing unparalleled spatiotemporal control over biological processes.[6,123,124] Exemplified by the success of photodynamic therapy (PDT), where light is combined with exogenously delivered sensitizers to trigger spatiotemporally controlled generation of reactive oxygen species, further phototherapeutic approaches are foreseen to have profound implications on targeted therapies.[125,126,127] Amongst such emerging approaches, photoactivated chemotherapy (PACT) is receiving increasing attention.[128,129,130,131] PACT relies on the use of PPGs that transiently disable the biological activity of cytotoxic drugs (payload, cargo) through a specific covalent linkage. Light irradiation of such photoresponsive prodrugs triggers the release of the reactivated drug *via* bond cleavage (photo-uncaging). PACT may represent an alternative to PDT, and more importantly it has the promise of complementary action, where PDT fails (i.e., in hypoxic tumors). Despite its advantages and the availability of the directly transferable advanced technology of light delivery from PDT, clinical translation of PACT is still hindered by limitations of existing PPGs. Such limitations include UV light activation, poor water solubility, and the lack of potential for targeting.

Considerable effort has been made recenty to develop suitably hydrophilic PPGs activatable in the biologically benign red or near-infrared (NIR) range.[132,133] Further challenges are posed by the lack of specific targeting of PPG-drug conjugates.[68] Although photoactivated chemotherapy combines the beneficial effects of external radiation therapy and internal chemotherapy and offers excellent localization-precision in case of localized primary tumors treatment of dispersed, hard-to-localize multiple tumors, remains an enormous challenge, where an extra level of spatial control is needed.

B. Discussion of results

Most photocages are based on *o*-nitrobenzyl or coumarinylmethyl scaffolds. A serious limitation of these PPgs in biological or medical application is the need for phototoxic UV light with quite poor penetration potential to trigger their removal from the cargo. [134] Shifting the activation wavelength toward the visible range facilitates tissue penetration and diminishes phototoxicity. Current efforts to develop new photocages that can respond to visible light have resulted in various blue-light activatable PPGs based on coumarin, [135] quinolinium, [136] trimethyl-lock scaffolds, [137] ruthenium-based complexes. [138] Higher wavelength, i.e. green light, activatable photocages, however, are less common and mostly limited to BODIPY[139,140,141] derivatives.* These BODIPY photocages are known to have high absorption coefficients and photochemical quantum yields, but usually poor water solubility and cumbersome synthetic accessibility. Since PPGs and fluorescent probes have a lot in common, we applied our vast knowledge on fluorescent probes to the design of red-shift PPGs. Along these considerations, we designed a new class of cationic green-orange absorbing photocages by extending the π-system of an existing coumarin PPG scaffold with vinylpyridinium or vinylbenzothiazolium motifs (compounds 1-3 in Figure 17).[142] The compounds showed significantly red-shifted absorption maxima (i.e., between 480 and 540 nm) and molar absorption coefficients in the range of 30 000–40 000 $M^{-1}cm^{-1}$. Furthermore, the broad absorption bands allow for longer wavelength activation up to 600 nm. Uncaging efficiencies enabled efficient green-light triggered release of a caged model compound. These new photocages also showed exceptionally high two-photon cross sections, suggesting their use in two-photon uncaging experiments. This design strategy based on the π-extension of synthetically accessible PPGs with solubilizing motifs allows access to further water-soluble red-shifted photocages for various applications in life sciences.

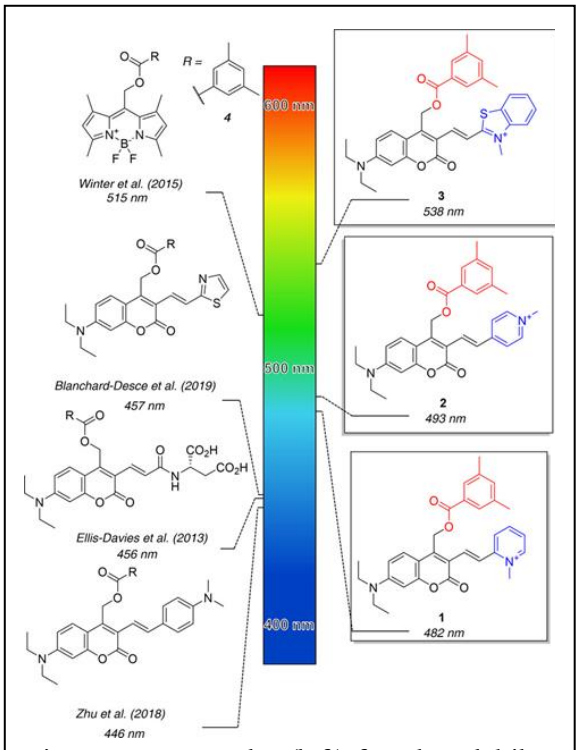

Figure 17. Examples (left) for photolabile coumarins and a benchmark BODIPY derivative and our π-extended photocages of this work (right)

* Just very recently our group and the research group of Stacko reported the synthesis and application of red, far-red, near IR activatable xanthenium and cyanine-based photocages see refs. 129, 130.

In addition to improving the spectral features of PPGs, their impact on chemical biology or medical applications such as photoactivated chemoterapy (PACT) could also benefit from the development of such improved photocages possessing specific targeting elements. In this respect, involvement of bioorthogonal chemistry seems reasonable, similarly to the highly specific installation of fluorescent probes onto biomolecules. There are a few notable examples in the literature on "clickable" photocages targeting various intracellular compartments. However, in these reports, click-chemistry (i.e., CuAAC) was employed to assemble the organelle-targeting photocage, rather than to serve as the targeting element itself.[143,144,145] To the best of our knowledge, such clickable photocages, where the clickable moiety is also the targeting element were not reported. Redefining the role of the clickable function, however, is rather considered as an incremental step toward advanced photocages. We therefore aimed for something more special and wished to add an extra twist to the story. On the basis of our extensive work on the development of bioorthogonally controlled fluorogenic probes, we hypothesized that a similar concept can be applied to modulate the photoresponsivity of photocages. We assumed that both fluorescence and the photodissociation step proceeds from the very same excited state that can be modulated e.g., by a tetrazine. This foreseen concept, termed "conditional photocaging", would render PPG-drug conjugates photoresponsive ("armed") solely by a chemical transformation of the modulating moiety in a specific bioorthogonal reaction. Provided that the bioorthogonal step is also the means of ligation of the PPG-drug conjugate to a target platform, implies that only specifically delivered drugs are liberated upon light exposure, while non-specifically bound or free constructs remain inactive. To prove our hypothesis, we sought for a synthetically easily accessible model compound that also allows visible light excitation. As seen, we have managed to shift the activation wavelength of coumarin-derived photocages by extending their π-system with electron withdrawing motifs. Parallel to this, we attempted to shift the wavelength of ultrafluorogenic coumarins developed by the Weissleder group,[64] by applying the π-extension approach. This latter approach allowed us to gain access to a large Stokes-shift (λ_{exc} 489 nm / λ_{em} 614 nm) coumarin probe (HELIOS-like 488Me) with good fluorogenicity (i.e., 30-fold), which complies the TBET principles for tetrazine quenching (Figure 18).[146] Part of this project we also identified a vinylene linked

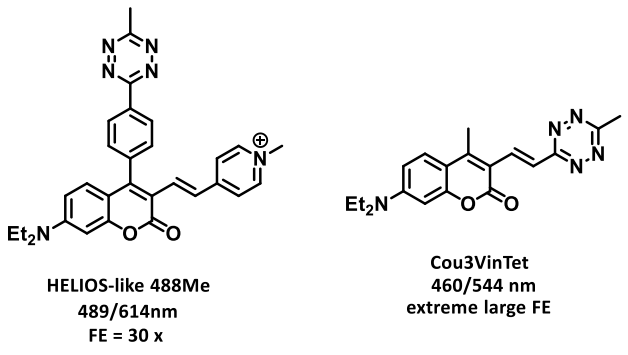

Figure 18. Structure of HELIOS-like 488Me and ultrafluorogenic, π-extended vinyltetrazin appendied coumarin probe, Cou3vinTet

coumarin-tetrazine that preserves ultrafluorogenic characteristics with considerably red shifted excitation and emission wavelengths.

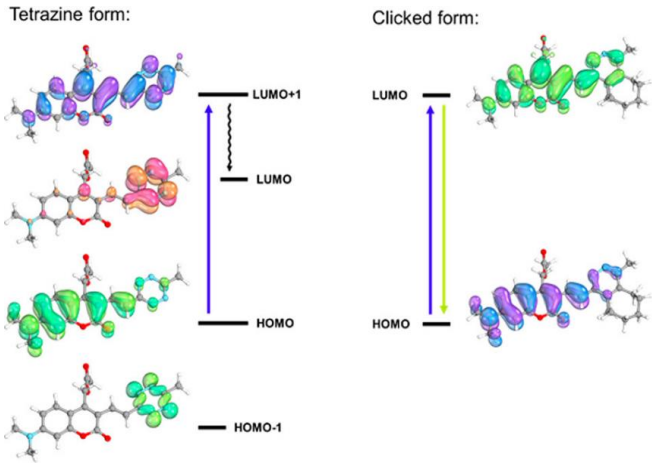

Figure 19. Low-lying excited states of the model tetrazine and its BCN-clicked product

Theoretical investigations revealed that that the vinylene linkage participates in the π-system of the chromophore, which explains the red-shifted absorbance. Furthermore, it was revealed that the S1 state of the vinylene-linked tetrazine-coumarin corresponds to the dark n → π* excitation of tetrazine (HOMO–1 → LUMO), while the S_2 state is predominantly formed by promoting an electron from the highest π orbital of the vinylcoumarin to the lowest-lying π* orbital of the tetrazine-vinylcoumarin system (HOMO → LUMO+1) (Figure 19). The probabilities of both the $S_0 \rightarrow S_2$ and the $S_0 \leftarrow S_2$ transitions are high, which suggests that the molecule gets into its S_2 state upon irradiation with blue light, followed by a rapid internal conversion to the dark S_1 and then to the ground state. After iEDDA reaction with BCN, the n → π* type state no longer exists and the π → π* state of the vinylcoumarin (HOMO → LUMO transition of the cyclooctyne-conjugated model compound) becomes the lowest singlet excited state enabling fluorescence. The photoreaction presumably also takes place on the S_2 surface, thus the presence of the tetrazine ring precludes both the reaction and the radiative decay of the excited state. The very same results were obtained with Cou3VinTet derived PPG, where a hydroxymethylene moiety was appended at the 4th position pf the coumarin.[147] Very importantly, in the "armed" form of this latter derivative accumulation of electron density in the excited state in a position adjacent to the carbon-payload bond was observed. Combining this filled orbital with the adjacent antibonding orbital weakens the carbon-payload bond, which in turn promotes departure of the payload.[148] In

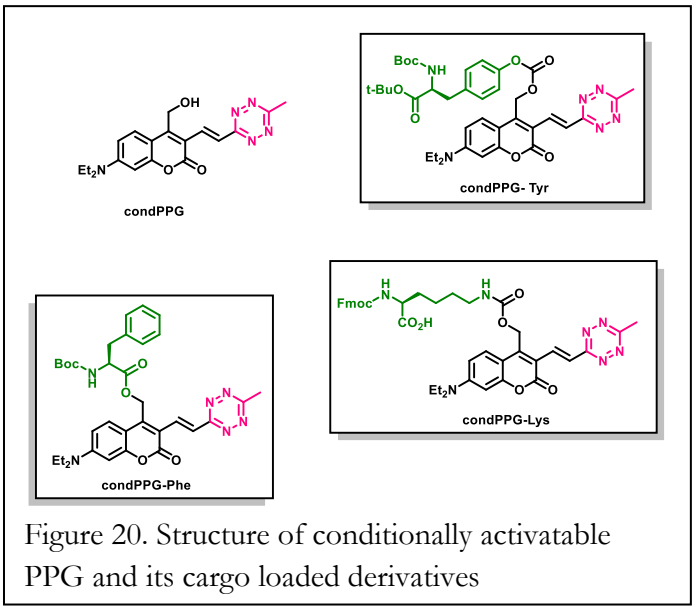

Figure 20. Structure of conditionally activatable PPG and its cargo loaded derivatives

light of these, we have synthesized condPPG and further conjugated it with three different amino acids (Phe, Lys and Tyr) as model caged molecules to readily access condPPG-cargo conjugates linked *via* ester (condPPG-Phe), carbamate (condPPG-Lys), and carbonate (condPPG-Tyr) linked species, respectively (Figure 20).

In accordance with our previous observations, absorption spectra of all derivatives were centered at around 475 nm (tetrazine form) with medium molar absorption coefficients (35–40 000 M^{-1} cm^{-1}). The conjugates also lived up to our expectations as they possessed practically zero fluorescence and uncaging efficiency in their tetrazine forms. Arming the PPG-conjugates in a reaction with BCN resulted in blue-shifted absorption maxima (around 445 nm) and a ca. 1000-fold increase in bright green emission intensity at around 535 nm. To our delight, blue-light (460 nm) irradiation of the "click - armed" forms led to the rapid release of all three amino acids. Uncaging efficiencies were good and excellent in case of Phe and Tyr conjugates attached to condPPG through carboxylate or carbonate moieties, respectively. When the cargo attachment was realized *via* carbamate linkage (Lys) the uncaging efficiency was a bit lower, but still within the acceptable range. Since this latter was found to be the most stable under physiological conditions, we applied the carbamate linkage in the more challenging live cell experiments. Although condPPG is inherently fluorogenic and would indicate the localization of the conjugates in cells, appearance of its fluorescence would not provide information about the uncaging process itself. Therefore, we loaded a fluorogenic rhodol[149] type of cargo onto it, *via* a self-immolative linker.[150] The well-established dimethylethylenediamine-carbamoyl self-immolative linker allows attachment of the rhodol through a carbamate linkage and provides sufficient spatial separation and fast release kinetics by suppressing recombination of the contact ion pairs formed primarily upon the photodissociation step. Further to this, the carbamoyl-derived rhodol is practically nonemissive. We were pleased to see that irradiation (activation) of the unarmed condPPG-rhodol conjugate with blue light did not lead to the release of the cargo (Figure 21). Also, dark-stabilities indicated no unwanted cargo release. When, however, photoresponsivity was armed in a click reaction with BCN, irradiation (activation) of the contruct at 488 nm resulted in the appearance of the cargo's bright orange fluorescence. Gratifyingly, blue-light triggered liberation of the rhodol led to

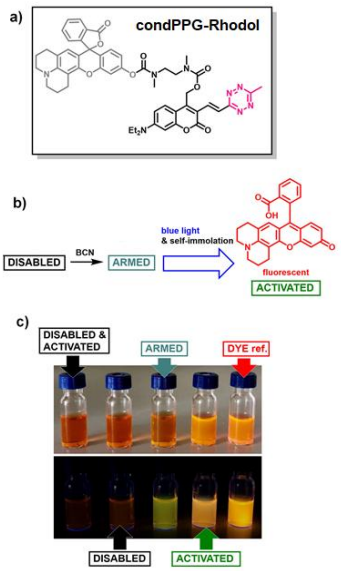

Figure 21. (a) Structure of condPPG-rhodol (b) scheme for the conditional uncaging and (c) photographs of the samples under ambient and UV light.

an overall 1000× increase of fluorescence intensity at the rhodol channel (λ_{exc} = 515 nm) following 15 min of irradiation. We applied this condPPG-Rhodol construct to demonstrate our conceptually novel conditionally activatable photocage system in live cells. We chose a BCN derivatized mitochondria targeting platform TPP-BCN to deliver the complementary bioorthogonal function into cells. Confocal fluorescence microscopy imaging of A-431 (skin cancer) cells either with or without pretreatment with TPP-BCN was used to demonstrate conditional uncaging. Image analyses revealed a clear colocalization of condPPG-Rhodol with MitoTracker Deep Red in case of cells that were pretargeted with TPP-BCN and treated with the condPPG-Rhodol construct, confirming successful bioorthogonal-targeting of the photocage inside the mitochondria. It can also be seen that the green emission of the coumarin upon excitation with the blue laser (488 nm) is only visible in the case of pretargeting, demonstrating the fluorogenicity of the coumarin photocage upon bioorthogonal conjugation. Contrary to this, no coumarin emission could be detected in the absence of TPP-BCN. An interesting and notable addition to the results is that no intracellular signal at the coumarin channel could be detected either, when the cells were treated with preassembled TPP-condPPG-Rhod, suggesting non-permeability of this construct and highlighting the importance of the 2-step assembly of active species inside cells. Following confirmation of the highly specific targeted delivery of condPPG-Rhodol to mitochondria, live-cell photouncaging of rhodol was triggered either by the built-in blue metal halide lamp of the microscope (excitation: 460-500 nm) or the blue excitation laser (488 nm). In case of cells treated both with TPP-BCN and condPPG-Rhodol bright fluorescence at the rhodol channel appeared mostly around the mitochondria. In contrast, cells treated with conPPG-Rhodol alone, only a small intensity fluorescence dispersed throughout the cells appeared at the rhodol channel (Figure 22). By contrast, the cells pretargeted with TPP-BCN displayed bright fluorescence

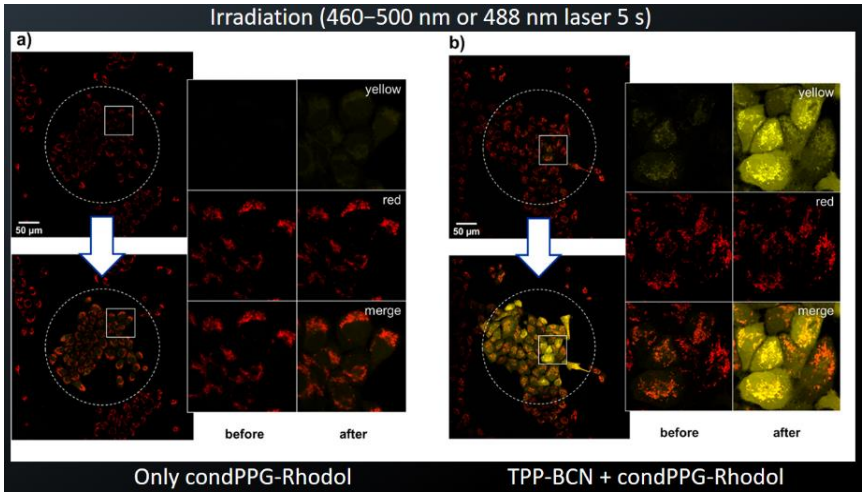

Figure 22. Tile scan experiments before and after irradiation of the central area (a) Cells treated only with tetrazine condPPG-Rhodol (b) cells pretargeted with TPP-BCN then treated with condPPG-Rhodol. The colors refer to the corresponding emission channels (yellow: rhodol, red: MitoTracker Deep Red)

after irradiation that is mostly located inside the mitochondria. These experiments provided evidence for the conditionally activatable PPG concept and confirmed that the confined irradiation area combined with the subcellular pretargeting can serve as dual control for highly localized manipulation as demonstrated by our fluorogenic click and uncage platform.

C. Evaluation of results and future directions

Several phototherapeutic approaches emerged lately, and these are foreseen to have profound implications on targeted therapies. Expanding the photolabile protecting group toolbox with visible light activatable, water-soluble scaffolds truly facilitate clinical translation of photoactivated chemotherapy. Besides, chromatically orthogonal PPGs enabling wavelength dependent activation of multiple functions / ligands allow photopharmacology to advance to a next level.

The excellent localization-precision of light offer remarkable targeting capabilities, but only in cases when the target itself can be localized e.g., solid tumors. Selective treatment of multiple, dispersed tumor cells, however, remains an enormous challenge, highlighting the need for advanced targeting mechanisms. The increasing number of recent examples where photoresponsivity is combined with bioorthogonal chemistry proved that the role of the bioorthogonal step has gone way beyond a simple ligation method. In these examples the bioorthogonal reaction has an added feature of being responsible for the activation of photoresponsivity. This combination of covalent targeting and activation of photoresponsivity allow to gain an even higher spatiotemporal control over light-triggered processes. Future research directions definitely involve the development of novel mechanisms that allow for such a combination of bioorthogonal ligation and photoresponsivity in the biologically more benign spectral range. Important to note that such directions are not limited to the bioorthogonal modulation of photoresponsivity, but also can be extended to light triggered activation of transiently disabled bioorthogonal functions. These are especially promising approaches in combination with drugs disabled by a cleavable complementary bioorthogonal function (i.e., click-to-release TCO). Notable pioneering examples in this field are the approaches that rely on the photocontrolled activation of tetrazines or recent results on photocatalytically activated tetrazines.[151,152]

REFERENCES

[1] J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13. https://doi.org/10.1038/nchembio0605-13

[2] H. C. Hang, C. Yu, D. L. Kato, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U S A.* **2003**, *100*, 14846. https://doi.org/10.1073/pnas.2335201100

[3] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007. https://doi.org/10.1126/science.287.5460.2007

[4] R. E. Bird, S. A. Lemmel, X. Yu, Q. A. Zhou *Bioconjugate Chem.* **2021**, *32*, 2457. https://doi.org/10.1021/acs.bioconjchem.1c00461

[5] Z. Yu, L. Y. Ho, Q. Lin, *J. Am. Chem. Soc.* **2011** *133*, 11912. https://doi.org/10.1021/ja204758c

[6] B. D. Fairbanks, L. J. Macdougall, S. Mavila, J. Sinha, B. E. Kirkpatrick, K. S. Anseth, C. N. Bowman, *Chem. Rev.* **2021**, *121*, 6915. https://doi.org/10.1021/acs.chemrev.0c01212

[7] G. S. Kumar, Q. Lin, *Chem. Rev.* **2021**, *121*, 6991. https://doi.org/10.1021/acs.chemrev.0c00799

[8] M. F. Debets, C. W. van der Doelen, F. P. Rutjes, F. L. van Delft, *ChemBioChem* **2010**, *11*, 1168. https://doi:10.1002/cbic.201000064

[9] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596. https://doi.org/10.1002/1521-3773(20020715)41:14%3C2596::AID-ANIE2596%3E3.0.CO;2-4

[10] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67* 3057. https://doi.org/10.1021/jo011148j

[11] D. S. del Amo, W. Wang, H. Jiang, C. Besanceney, A. C. Yan, M. Levy, Y. Liu, F. L. Marlow, P. Wu, *J. Am. Chem. Soc.* **2010**, *132*, 16893. https://doi.org/10.1021/ja106553e

[12] S. Li, L. Wang, F. Yu, Z. Zhu, D. Shobaki, H. Chen, M. Wang, J. Wang, G. Qin, U. J. Erasquin, L. Ren, Y. Wang, C. Cai, C. *Chem Sci.* **2017**, *8*, 2107. https://doi.org/10.1039/C6SC02297A

[13] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. A. Chem. Soc.* **2004** *126*, 15046. https://doi.org/10.1021/ja044996f

[14] J. Dommerholt, F. P. J. T. Rutjes, F. L. van Delft, *Top. Curr. Chem.* **2016**, *374*, 16. https://doi.org/10.1007/s41061-016-0016-4

[15] M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518. https://doi.org/10.1021/ja8053805

[16] N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjugate Chem.* **2008**, *19*, 2297. https://doi.org/10.1021/bc8004446

[17] A. C. Knall, C. Slugovc, *Chem. Soc. Rev.* **2013**, *42*, 5131. https://doi.org/10.1039/C3CS60049A

[18] J. E. Hoffmann, T. Plass, I. Nikić, I. V. Aramburu, C., Koehler, H. Gillandt, E. A. Lemke, C. Schultz, *Chem. Eur. J.* **2015**, *21*, 12266. https://doi.org/10.1002/chem.201501647

[19] M. M. A. Mitry, F. Greco, H. M. I. Osborn, *Chem. Eur. J.* **2023**, *29*, e202203942. https://doi.org/10.1002/chem.202203942

[20] S. L. Scinto, D. A. Bilodeau, R. Hincapie, W. Lee, S. S. Nguyen, M. Xu, C. W. am Ende, M. G. Finn, K. Lang, Q. Lin, J. P. Pezacki, J. A. Prescher, M. S. Robillard, J. M. Fox, *Nat. Rev. Methods Primers* **2021**, *1*, 30. https://doi.org/10.1038/s43586-021-00028-z

[21] N. K. Devaraj, *ACS Cent. Sci.* **2018**, *4*, 952. https://doi.org/10.1021/acscentsci.8b00251

[22] X. Ji, Z. Pan, B. Yu, L. K. de La Cruz, Y. Zheng, B. Ke, B. Wang, *Chem. Soc. Rev.* **2019**, *48*, 1077. https://doi.org/10.1039/C8CS00395E

[23] R. Rossin, S. M. J. van Duijnhoven, W. ten Hoeve, H. M. Janssen, L. H. J. Kleijn, F. J. M. Hoeben, R. M. Versteegen, M. S. Robillard, *Bioconjugate Chem.* **2016**, 27, 1697. https://doi.org/10.1021/acs.bioconjchem.6b00231

[24] J. Ko, M. Wilkovitsch, J. Oh, R. H. Kohler, E. Bolli, M. J. Pittet, C. Vinegoni, D. B. Sykes, H. Mikula, R. Weissleder, C. T. Carlson, *Nat. Biotechnol.* **2022**, *40*, 1654. https://doi:10.1038/s41587-022-01339-6

[25] A. H. A. M. van Onzen, R. M. Versteegen, F. J. M. Hoeben, I. A. W. Filot, R. Rossin, T. Zhu, J. Wu, P. J. Hudson, H. M. Janssen, W. Hoeve, M. S. Robillard, *J. Am. Chem. Soc.* **2020**, *142*, 10955. https://doi.org/10.1021/jacs.0c00531

[26] ClinicalTrials.gov Identifier: NCT04106492.

[27] ClinicalTrials.gov Identifier: NCT03682796.

[28] M. L. W. J. Smeenk, J. Agramunt, K. M. Bonger, *Curr. Opin. Chem. Biol.* **2021**, *60*, 79. https://doi.org/10.1016/j.cbpa.2020.09.002

[29] Q. Wu, K. Y. Zhang, P. Dai, H. Zhu, Y. Wang, L. Song, L. Wang, S. Liu, Q. Zhao, W. Huang, *J. Am. Chem. Soc.* **2020**, *142*, 1057. https://doi.org/10.1021/jacs.9b12191

[30] I. Nikić, T. Plass, O. Schraidt, J. Szymański, J. A. G. Briggs, C. Schultz, E. A. Lemke, *Angew. Chem. Int. Ed.* **2014**, *53*, 2245. https://doi.org/10.1002/anie.201309847

[31] U. Reisacher, D. Ploschik, F. Rönicke, G. B. Cserép, P. Kele, H.-A. Wagenknecht, *Chem. Sci.* **2019**, *10*, 4032. https://doi.org/10.1039/c8sc05588b

[32] M. Grammel, H. C. Hang, *Nat. Chem. Biol.* **2013**, *9*, 475. https://doi.org/10.1038/nchembio.1296

[33] U. Reisacher, B. Groitl, R. Strasser, G. B. Cserép, P. Kele, H-A. Wagenknecht, *Bioconjugate Chem.* **2019**, *30*, 1773. https://doi.org/10.1021/acs.bioconjchem.9b00295

[34] K. K. Palaniappan, C. R. Bertozzi, *Chem. Rev.* **2016**, *116*, 14277. https://doi.org/10.1021/acs.chemrev.6b00023

[35] P. Agarwal, B. J. Beahm, P. Shieh, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2015**, *54*, 11504. https://doi.org/10.1002/anie.201504249

[36] P. Shieh, V. T. Dien, B. J. Beahm, J. M. Castellano, T. Wyss-Coray, C. R. Bertozzi, CalFluors: A Universal Motif for Fluorogenic Azide Probes across the Visible Spectrum. *J. Am. Chem. Soc.* **2015**, *137*, 7145. https://doi.org/10.1021/jacs.5b02383

[37] J. Schoch, M. Wiessler, A. Jäschke, *J. Am. Chem. Soc.* **2010**, *132*, 8846. https://doi.org/10.1021/ja102871p

[38] D. Ploschik, F. Rönicke, H. Beike, R. Strasser, H-A. Wagenknecht, *ChemBioChem* **2018**, *19*, 1949. https://doi.org/10.1002/cbic.201800354

[39] J. Yang, J. Šečkutė, C. M. Cole, N. K. Devaraj, *Angew. Chem. Int. Ed.* **2012**, *51*, 7476. https://doi.org/10.1002/anie.201202122

[40] A. Neef, C. Schultz, *Angew. Chem. Int. Ed.* **2009**, *48*, 1498. https://doi.org/10.1002/anie.200805507

[41] K. Lang, J. W. Chin, *ACS Chem. Biol.* **2014**, *9*, 16. https://doi.org/10.1021/cb4009292

[42] T. Plass, S. Milles, C. Koehler, C. Schultz, E. A. Lemke, *Angew. Chem. Int. Ed.* **2011**, *50*, 3878. https://doi.org/10.1002/anie.201008178

[43] P. Kumar, W. Huang, D. Shukhman, F. M. Camarda, S. T. Laughlin, *Tetrahedron Lett.* **2019**, *60*, 1476. https://doi.org/10.1016/j.tetlet.2019.04.046

[44] A. Espinasse, X. Wen, J. D. Goodpaster, E. E. Carlson, *ACS Chem. Biol.* **2020**, *15*, 1252. https://doi.org/10.1021/acschembio.9b01024

[45] J. Chin, *Nature* **2017**, *550*, 53. https://doi.org/10.1038/nature24031

[46] T, Tamura, I. Hamachi, *J. Am. Chem. Soc.* **2019**, *141*, 2782. https://doi.org/10.1021/jacs.8b11747

[47] Y. Zhang, K.-Y. Park, K.F. Suazo, M.D. Distefano, *Chem. Soc. Rev.* **2018**, *47*, 9106. https://doi.org/10.1039/C8CS00537K

[48] D.G. Rawale, K. Thakur, S. R. Adusumalli, V. Rai, *Eur. J. Org. Chem.* **2019**, *40*, 6749. https://doi.org/10.1002/ejoc.201900801

[49] H. E. Murrey, J. C. Judkins, C. W. am Ende, T. E. Ballard, Y. Fang, K. Riccardi, L. Di, E. R. Guilmette, J. W. Schwartz, J. M. Fox, D. S. Johnson, *J. Am. Chem. Soc.* **2015**, *137*, 11461. https://doi.org/10.1021/jacs.5b06847

[50] Y. M. Sigal, R. Zhou, X. Zhuang, *Science* **2018**, *361*, 880. https://doi.org/10.1126/science.aau1044

[51] L. Wang, M. S. Frei, A. Salim, K. Johnsson, *J. Am. Chem. Soc.* **2019**, *141*, 2770. https://doi.org/10.1021/jacs.8b11134

[52] M Monici, *Biotech. Annu. Rev.* **2005**, *11*, 227. https://doi.org/10.1016/S1387-2656(05)11007-2

[53] Y. Hori, K. Kikuchi, *Curr. Opin. Chem. Biol.* **2013**, *17*, 644. https://doi.org/10.1016/j.cbpa.2013.05.015

[54] L. Wang, M. Tran, E. D'Este, J. Roberti, B. Koch, L. Xue, K. Johnsson, *Nat. Chem.* **2020**, *12*, 165. https://doi.org/10.1038/s41557-019-0371-1

[55] E. Kozma, P. Kele, *Org. Biomol. Chem.* **2019**, *17*, 215. https://doi.org/10.1039/C8OB02711K

[56] S. Liu, P. Hoess, J. Ries, *Annu. Rev. Biophys.* **2022** *51*, 301. https://doi.org/10.1146/annurev-biophys-102521-112912

[57] P. An, Q. Lin, *Org. Biomol. Chem.* **2018**,*16*, 5241. https://doi.org/10.1039/C8OB01404C

[58] X. Shang, R. Lai, X. Song, H. Li, W. Niu, J. Guo, *Bioconjugate Chem.* **2017**, *28*, 2859. https://doi.org/10.1021/acs.bioconjchem.7b00562

[59] F. Friscourt, C. J. Fahrni, G-J. Boons, *J. Am. Chem. Soc.* **2012**, *134*, 18809. https://doi.org/10.1021/ja309000s

[60] J. C. Jewett, C. R. Bertozzi, *Org. Lett.* **2011**, *13*, 5937. https://doi.org/10.1021/ol2025026

[61] Z. Zhou, C. J. Fahrni, *J. Am. Chem. Soc.* **2004**, *126*, 8862. https://doi.org/10.1021/ja049684r

[62] C. Favre, F. Friscourt, *Org. Lett.* **2018**, *20*, 4213. https://doi.org/10.1021/acs.orglett.8b01587

[63] K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603. https://doi.org/10.1021/ol047955x

[64] L. G. Meimetis, J. C. T. Carlson, R. J. Giedt, R. H. Kohler, R. Weissleder, *Angew. Chem. Int. Ed.* **2014**, *53*, 7531. https://doi.org/10.1002/anie.201403890

[65] J. C. T. Carlson, L. G. Meimetis, S. A. Hilderbrand, R. Weissleder, *Angew. Chem. Int. Ed.* **2013**, *52*, 6917. https://doi.org/10.1002/anie.201301100

[66] G. Linden, L. Zhang, F. Pieck, U. Linne, D. Kosenkov, R. Tonner, O. Vázquez, *Angew. Chem. Int. Ed.* **2019**, *58*, 12868. https://doi.org/10.1002/anie.201907093

[67] J. Qiao, F. Tian, Y. Deng, Y. Shang, S. Chen, E. Chang, J. Yao, *Theranostics* **2020**, *10*, 5305. https://doi.org/10.7150/thno.42445

[68] E. Kozma, M. Bojtár, P. Kele, *Angew. Chem. Int. Ed.* **2023**, *62*, e202303198. https://doi:10.1002/anie.202303198

[69] B. Pinto-Pacheco, W. P. Carbery, S. Khan, D. B. Turner, D. Buccella, *Angew. Chem. Int. Ed.* **2020**, *59*, 22140. https://doi.org/10.1002/anie.202008757

[70] N. Devaraj, S. Hilderbrand, R. Upadhyay, R. Mazitschek, R. Weissleder, *Angew. Chem. Int. Ed.* **2010**, *49*, 2869. https://doi.org/10.1002/anie.200906120

[71] A. Wieczorek, P. Werther, J. Euchner, R. Wombacher, *Chem. Sci.* **2017**, *8*, 1506. https://doi.org/10.1039/C6SC03879D

[72] P. Werther, K. Yserentant, F. Braun, K. Grußmayer, V. Navikas, M. Yu, Z. Zhang, M. J. Ziegler, C. Mayer, A. J. Gralak, M. Busch, W. Chi, F. Rominger, A. Radenovic, X. Liu, E. A. Lemke, T. Buckup, D-P. Herten, R. Wombacher, *ACS Centr. Sci.* **2021**, *7*, 1561. https://doi.org/10.1021/acscentsci.1c00703

[73] A. Herner, I. Nikić, M. Kállay, E. A. Lemke, P. Kele, *Org. Biomol. Chem.* **2013**, *11*, 3297. https://doi.org/10.1039/C3OB40296G

[74] A. Herner, G. E. Girona, I. Nikić, M. Kállay, E. A. Lemke, P. Kele, *Bioconjugate Chem.* **2014**, *25*, 1370. https://doi.org/10.1021/bc500235p

[75] O. Demeter, E. A. Fodor, M. Kállay, G. Mező, K. Németh, P. T. Szabó, P. Kele, *Chem. Eur. J.* **2016**, *22*, 6382. https://doi.org/10.1002/chem.201504939

[76] O. Demeter, A. Kormos, C. Koehler, G. Mező, K. Németh, E. Kozma, L. B. Takács, E. A. Lemke, P. Kele, *Bioconjugate Chem.* **2017**, *28*, 1552. https://doi.org/10.1021/acs.bioconjchem.7b00178

[77] A. Kormos, C. Koehler, E. A. Fodor, Z. R. Rutkai, M. E. Martin, G. Mező, E. A. Lemke, P. Kele, *Chem. Eur. J.* **2018**, *24*, 8841. https://doi.org/10.1002/chem.201800910

[78] E. Kozma, G. Estrada Girona, G. Paci, E. A. Lemke, P. Kele, *Chem. Commun.* **2017**, *53*, 6696. https://doi.org/10.1039/C7CC02212C

[79] H. Ito, Y. Kawamata, M. Kamiya, K. Tsuda-Sakurai, S. Tanaka, T. Ueno, T. Komatsu, K. Hanaoka, S. Okabe, M. Miura, Y. Urano, *Angew. Chem. Int. Ed.* **2018**, *57*, 15702. https://doi.org/10.1002/anie.201808670

[80] P. Werther, K. Yserentant, F. Braun, N. Kaltwasser, C. Popp, M. Baalmann, D.-P. Herten, R. Wombacher, *Angew. Chem. Int. Ed.* **2020**, *59*, 804. https://doi.org/10.1002/anie.201906806

[81] T. Iwaoka, M. Kondo, *Bull. Chem. Soc. Jpn.* **1974**, *47*, 980. https://doi.org/10.1246/bcsj.47.980

[82] R. W. Redmond, J. N. Gamlin, *Photochem. Photobiol.* **1999**, *70*, 391. https://doi.org/10.1111/j.1751-1097.1999.tb08240.x

[83] T. Manju , N. Manoj , A. M. Braun and E. Oliveros, *Photochem. Photobiol. Sci.* **2012**, *11*, 1744. https://doi.org/10.1039/C2PP25244A

[84] C. Liu , X. Jiao , Q. Wang , K. Huang , S. He , L. Zhao and X. Zeng , *Chem. Commun.* **2017**, *53*, 10727. https://doi.org/10.1039/C7CC06220F

[85] A. Kormos, D. Kern, A. Egyed, B. Söveges, K. Németh, P. Kele, *Chem. Commun.* **2020**, *56*, 5425. https://doi.org/10.1039/D0CC01512A

[86] A. Kormos, A. Egyed, J. M. Olvany, Á. Szatmári, A. Biró, Zs. Csorba, P. Kele, K. Németh, *Chemosensors* **2022**, *10*, 37. https://doi.org/10.3390/chemosensors10010037

[87] G. Knorr, E. Kozma, A. Herner, E. A. Lemke, P. Kele, *Chem. Eur. J.* **2016**, *22*, 8972. https://doi.org/10.1002/chem.201600590

[88] G. Knorr, E. Kozma, J. M. Schaart, K. Németh, Gy. Török, P. Kele, *Bioconjugate Chem.* **2018**, *29*, 1312. https://doi.org/10.1021/acs.bioconjchem.8b00061

[89] E. Albitz, K. Németh, G. Knorr, P. Kele, *Org. Biomol. Chem.* **2023**, *21*, 7358. https://doi.org/10.1039/D3OB01204B

[90] E. Albitz, D. Kern, A. Kormos, M. Bojtár, G. Török, A. Biró, Á. Szatmári, K. Németh, P. Kele, *Angew. Chem. Int. Ed.* **2022**, *61*, e202111855. https://doi.org/10.1002/anie.202111855

[91] S. Basu, L. Needham, D. Lando, E. J. R. Taylor, K. J. Wohlfahrt, D. Shah, W. Boucher, Y. L. Tan, L. E. Bates, O. Tkachenko, J. Cramard, B. C. Lagerholm, C. Eggeling, B. Hendrich, D. Klenerman, S. F. Lee, E. D. Laue, *Nat. Commun.* **2018**, *9*, 2520. https://doi.org/10.1038/s41467-018-04486-0

[92] D. Ganz, D. Harijan, H-A. Wagenknecht, *RSC Chem. Biol.* **2020**, *1*, 86. https://doi.org/10.1039/D0CB00047G

[93] P. Kele, G. Mezö, D. Achatz, O. Wolfbeis, *Angew. Chem. Int. Ed.* **2009**, *48*, 344. https://doi.org/10.1002/anie.200804514

[94] A. Aktalay, R. Lincoln, L. Heynck, M. A. do R. B. F. Lima, A. N. Butkevich, M. L. Bossi, S. W. Hell, *ACS Centr. Sci.* **2023**, *9*, 1581. https://doi.org/10.1021/acscentsci.3c00746

[95] N. Aloush, T. Schvartz, A. I. König, S. Cohen, E. Brozgol, B. Tam, D. Nachmias, O. Ben-David, Y. Garini, N. Elia, E. Arbely, *Sci. Rep.* **2018**, *8*, 14527. https://doi.org/10.1038/s41598-018-32824-1

[96] T. S. Mihaila, C. Bäte, L. M. Ostersehlt, J. K. Pape, J. Keller-Findeisen, S. J. Sahl, S. W. Hell, *Proc. Natl. Acad. Sci U.S.A.* **2022**, *119*, e2201861119. https://doi.org/10.1073/pnas.2201861119

[97] D. Cervettini, S. Tang, S. D. Fried, J. C. W. Willis, L. F. H. Funke, L. J. Colwell, J W. Chin, *Nat Biotech.* **2020**, *38*, 989. https://doi.org/10.1038/s41587-020-0479-2

[98] W. H. Schmied, Z. Tnimov, C. Uttamapinant, C. D. Rae, S. D. Fried, J. W. Chin, *Nature* **2018**, *564*, 444. https://doi.org/10.1038/s41586-018-0773-z

[99] C. D. Reinkemeier, E. A. Lemke, *Cell* **2021**, *184*, 4886.e21. https://doi.org/10.1016/j.cell.2021.08.001

[100] K. Chenoweth, D. Chenoweth, W. A. Goddard III, *Org. Biomol. Chem.* **2009**, *7*, 5255. https://doi.org/10.1039/B911482C

[101] B. R. Varga, M. Kállay, K. Hegyi, S. Béni, P. Kele, *Chem. Eur. J.* **2012**, *18*, 822. https://doi.org/10.1002/chem.201102329

[102] A. Krebs, J. Odenthal, H. Kimling, *Tetrahedron Lett.* **1975**, *16*, 4663. https://doi.org/10.1016/S0040-4039(00)91046-3

[103] H. Meier, M. Layer, A. Zetzsche, *Chem.-Ztg.* **1974**, *98*, 460.

[104] M. Merkel, S. Arndt, D. Ploschik, G. B. Cserép, U. Wenge, P. Kele, H-A. Wagenknecht, *J. Org. Chem.* **2016**, *81*, 7527. https://doi.org/10.1021/acs.joc.6b01205

[105] 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, *Nat. Mater.* **2015**, *14*, 125. https://doi.org/10.1038/nmat4118

[106] R. B. Quast, B. Ballion, M. Stech, A. Sonnabend, B. R. Varga, D. A. Wüstenhagen, P. Kele, S. M. Schiller, S. Kubick, *Sci. Rep.* **2016**, *6*, 34048. https://doi.org/10.1038/srep34048

[107] C. Stubinitzky, G. B. Cserép, E. Bätzner, P. Kele, H-A. Wagenknecht, *Chem. Commun.* **2014**, *50*, 11218. https://doi.org/10.1039/C4CC02855D

[108] J. Dommerholt, S. Schmidt, R. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl, F. L. van Delft, *Angew. Chem. Int. Ed.* **2010**, *49*, 9422. https://doi.org/10.1002/anie.201003761

[109] A. Borrmann, S. Milles, T. Plass, J. Dommerholt, J. M. M. Verkade, M. Wießler, C. Schultz, J. C. M. van Hest, F. L. van Delft, E. A. Lemke, *ChemBioChem* **2012**, *13*, 2094. https://doi.org/10.1002/cbic.201200407

[110] S. Hilderbrand, B. R. Varga, R. Weissleder, Cyclooctenes for Bioorthogonal Reactions, WO 2016025480 (A1), **2016**.

[111] E. Kozma, I. Nikić, B. R. Varga, I. V. Aramburu, J. H. Kang, O. T. Fackler, E. A. Lemke, P. Kele, *ChemBioChem* **2016**, *17*, 1518. https://doi.org/10.1002/cbic.201600284

[112] G. B. Cserép, O. Demeter, E. Bätzner, M. Kállay, H-A. Wagenknecht, P. Kele, *Synthesis* **2015**, *47*, 2738. https://doi.org/10.1055/s-0034-1380721

[113] V. Šlachtová, S. Bellová, A. La-Venia, J. Galeta, M. Dračínský, K. Chalupský, A. Dvořáková, H. Mertlíková-Kaiserová, P. Rukovanský, R. Dzijak, M. Vrabel, *Angew. Chem. Int. Ed.* **2023**, *62*, e202306828. https://doi.org/10.1002/anie.202306828

[114] E. Decuypère, L. Plougastel, D. Audisio, F. Taran, *Chem. Commun.* **2017**, *53*, 11515. https://doi.org/10.1039/C7CC06405E

[115] G. B. Cserép, K. Németh, Á. Szatmári, F. Horváth, T. Imre, K. Németh, P. Kele, *Synthesis* **2022**, *54*, A. https://doi.org/10.1055/a-1761-4672

[116] X.-W. Liang , Y. Zhao , X.-G. Si , M.-M. Xu , J.-H. Tan , Z.-M. Zhang , C.-G. Zheng , C. Zheng, Q. Cai , *Angew. Chem., Int. Ed.* **2019**, *58*, 14562. https://doi.org/10.1002/anie.201908284

[117] C. J. F. Cole, L. Fuentes, S. A. Snyder, *Chem. Sci.* **2020**, *11*, 2175. https://doi.org/10.1039/C9SC05738B

[118] Gy. Török, G. B. Cserép, A. Telek, D. Arany, M. Váradi, L. Homolya, M. Kellermayer, P. Kele, K. Németh, *Methods Appl. Fluoresc.* **2021**, *9*, 015006. https://doi.org/10.1088/2050-6120/abb363

[119] J. Tu, D. Svatunek, S. Parvez, A. C. Liu, B. J. Levandowski, H. J. Eckvahl, R. T. Peterson, K. N. Houk, R. M. Franzini, *Angew. Chem. Int. Ed.* **2019**, *58*, 9043. https://doi.org/10.1002/anie.201903877

[120] Y. Chen, K-L. Wu, J. Tang, A. Loredo, J. Clements, J. Pei, Z. Peng, R. Gupta, X.Fang, H. Xiao, *ACS Chem. Biol.* **2019** *14*, 2793. https://doi.org/10.1021/acschembio.9b00678

[121] Á. Szatmári, G. B. Cserép, T. Á. Molnár, B. Söveges, A. Biró, G. Várady, E. Szabó, K. Németh, P. Kele, *Molecules* **2021**, *26*, 4988. https://doi.org/10.3390/molecules26164988

[122] I. Nikić, G. Estrada Girona, J. H. Kang, G. Paci, S. Mikhaleva, C. Koehler, N. V. Shymanska, C. Ventura Santos, D. Spitz, E. A. Lemke, *Angew. Chem. Int. Ed.* **2016**, *55*, 16172. https://doi.org/10.1002/anie.201608284

[123] K. Hüll, J. Morstein, D. Trauner, *Chem. Rev.* **2018**, *118*, 10710. https://doi.org/10.1021/acs.chemrev.8b00037

[124] B. Huang, H. Babcock, X. Zhuang, *Cell* **2010**, *143*, 1047. https://doi.org/10.1016/j.cell.2010.12.002

[125] S. Monro, K. L. Colón, H. Yin, J. Roque III, P. Konda, S. Gujar, R. P. Thummel, L. Lilge, C. G. Cameron, S. A. McFarland, *Chem. Rev.* **2019**, *119*, 797. https://doi.org/10.1021/acs.chemrev.8b00211

[126] B. M. Vickerman, E. M. Zywot, T. K. Tarrant, D. S. Lawrence, *Nat. Chem. Rev.* **2021**, *5*, 816. https://doi.org/10.1038/s41570-021-00326-w

[127] A. Bansal, Y. Zhang, *Acc. Chem. Res.* **2014**, *47*, 3052. https://doi.org/10.1021/ar500217w

[128] H. Xiong, Y. Xu, B. Kim, H. Rha, B. Zhang, M. Li, G. F. Yang, J. S. Kim, *Chem* **2023**, *9*, 29. https://doi.org/10.1016/j.chempr.2022.11.007

[129] S. Bonnet, *Dalton Trans.* **2018**, *47*, 10330. https://doi.org/10.1039/C8DT01585F

[130] H. Yu, J. Li, D. Wu, Z. Qiu, Y. Zhang, *Chem. Soc. Rev.* **2010**, *39*, 464. https://doi.org/10.1039/B901255A

[131] L. Bretin, Y. Husiev, V. Ramu, L. Zhang, M. Hakkennes, S. Abyar, A. C. Johns, S. E. Le Dévédec, T. Betancourt, A. Kornienko, S. Bonnet, *Angew. Chem. Int. Ed.* **2023**, e202316425. https://doi.org/10.1002/anie.202316425

[132] A. Egyed, K. Németh, T. Á. Molnár, M. Kállay, P. Kele, M. Bojtár, *J. Am. Chem. Soc.* **2023**, *145*, 4026. https://doi.org/10.1021/jacs.2c11499

[133] H. Janeková, H. C. Friedman, M. Russo, T. Ahmed, M. Zyberaj, S. Hua, A. V. Sica, J. R. Caram, P. Stacko, *ChemRxiv* **2023** https://doi.org/10.26434/chemrxiv-2023-c81hj

[134] P. Klán, T. Šolomek, C. G. Bochet, A. Blanc, R. Givens, M. Rubina, V. Popik, A. Kostikov, J. Wirz, *Chem. Rev.* **2013**, *113*, 119. https://doi.org/10.1021/cr300177k

[135] M. Klausen, V. Dubois, G. Clermont, C. Tonnelé, F. Castet, M. Blanchard-Desce, *Chem. Sci.* **2019**, *10*, 4209. https://doi.org/10.1039/C9SC00148D

[136] T. Narumi, K. Miyata, A. Nii, K. Sato, N. Mase, T. Furuta, *Org. Lett.* **2018**, *20*, 4178. https://doi.org/10.1021/acs.orglett.8b01505

[137] D. P. Walton, D. A. Dougherty, *J. Am. Chem. Soc.* **2017**, *139*, 4655. https://doi.org/10.1021/jacs.7b01548

[138] A. Li, C. Turro, J. J. Kodanko, *Acc. Chem. Res.* **2018**, *51*, 1415. https://doi.org/10.1021/acs.accounts.8b00066

[139] K. Sitkowska, B. L. Feringa, W. Szymański, *J. Org. Chem.* **2018**, *83*, 1819. https://doi.org/10.1021/acs.joc.7b02729

[140] J. A. Peterson, C. Wijesooriya, E. J. Gehrmann, K. M. Mahoney, P. P. Goswami, T. R. Albright, A. Syed, A. S. Dutton, E. A. Smith, A. H. Winter, *J. Am. Chem. Soc.* **2018**, *140*, 7343. https://doi.org/10.1021/jacs.8b04040

[141] T. Slanina, P. Shrestha, E. Palao, D. Kand, J. A. Peterson, A. S. Dutton, N. Rubinstein, R. Weinstain, A. H. Winter, P. Klán, *J. Am. Chem. Soc.* **2017**, *139*, 15168. https://doi.org/10.1021/jacs.7b08532

[142] M. Bojtár, A. Kormos, K. Kis-Petik, M. Kellermayer, P. Kele, *Org. Lett.* **2019**, *21*, 9410. https://doi.org/10.1021/acs.orglett.9b03624

[143] D. Kand, L. Pizarro, I. Angel, A. Avni, D. Friedmann-Morvinski, R. Weinstain, *Angew. Chem. Int. Ed.* **2019**, *58*, 4659. https://doi.org/10.1002/anie.201900850

[144] N. Wagner, M. Stephan, D. Höglinger, A. Nadler, *Angew. Chem. Int. Ed.* **2018**, *57*, 13339. https://doi.org/10.1002/anie.201807497

[145] A. Z. Suzuki, R. Sekine, S. Takeda, R. Aikawa, Y. Shiraishi, T. Hamaguchi, H. Okuno, H. Tamamura, T. Furuta, *Chem. Commun.* **2019**, *55*, 451. https://doi.org/10.1039/C8CC07981A

[146] E. Németh, G. Knorr, K. Németh, P. Kele, *Biomolecules* **2020**, *20*, 397. https://doi.org/10.3390/biom10030397

[147] M. Bojtár, K. Németh, F. Domahidy, G. Knorr, A. Verkman, M. Kállay, P. Kele, *J. Am. Chem. Soc.* **2020**, *142*, 15164. https://doi.org/10.1021/jacs.0c07508

[148] T. Šolomek, J. Wirz, P. Klán, *Acc. Chem. Res.* **2015**, *48*, 3064. https://doi.org/10.1021/acs.accounts.5b00400

[149] Y. M. Poronik, K. V. Vygranenko, D. Gryko, D. T. Gryko, *Chem. Soc. Rev.* **2019**, *48*, 5242. https://doi.org/10.1039/C9CS00166B

[150] A. Dal Corso, L. Pignataro, L. Belvisi, C. Gennari, *Chem. Eur. J.* **2019**, *25*, 14740. https://doi.org/10.1002/chem.201903127

[151] L. Liu, D. Zhang, M. Johnson, N. K. Devaraj, *Nat. Chem.* **2022**, *14*, 1078. https://doi.org/10.1038/s41557-022-00963-8

[152] A. Jemas, Y. Xie, J. E. Pigga, J. L. Caplan, C. W. am Ende, J. M. Fox, *J. Am. Chem. Soc.* **2022**, *144*, 1647. https://doi.org/10.1021/jacs.1c10390