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Biochemical Photoswitches

Light-Controlled Tools

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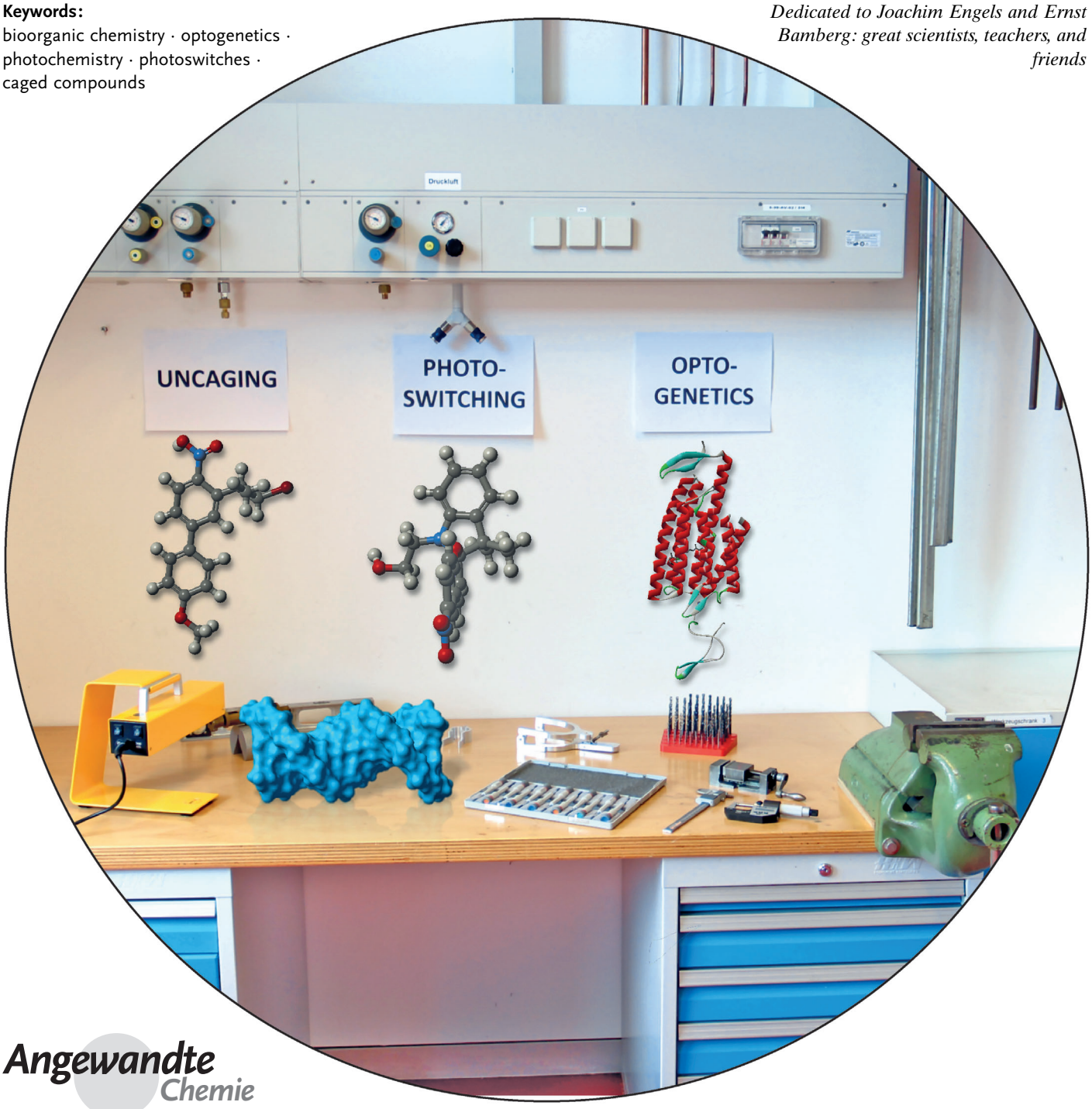
Dedicated to Joachim Engels and Ernst Bamberg: great scientists, teachers, and friends



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Spatial and temporal control over chemical and biological processes plays a key role in life, where the whole is often much more than the sum of its parts. Quite trivially, the molecules of a cell do not form a living system if they are only arranged in a random fashion. If we want to understand these relationships and especially the problems arising from malfunction, tools are necessary that allow us to design sophisticated experiments that address these questions. Highly valuable in this respect are external triggers that enable us to precisely determine where, when, and to what extent a process is started or stopped. Light is an ideal external trigger: It is highly selective and if applied correctly also harmless. It can be generated and manipulated with well-established techniques, and many ways exist to apply light to living systems—from cells to higher organisms. This Review will focus on developments over the last six years and includes discussions on the underlying technologies as well as their applications.

From the Contents

1. Introduction

Even though the idea of regulating processes with light is in no way new, recent years have seen a boom in improved technologies and new applications, as well as the development of one entirely new strategy: There are now three principle approaches for the regulation of light. One can use photolabile "protecting" groups to irreversibly trigger processes (uncaging, Section 2), photoswitches for reversible switching (Section 3), or genetically encoded light-responsive elements (optogenetics, Section 4). Each of these technologies has its advantages and disadvantages, and with the recent developments in each of the fields (for example, wavelength-selective photolabile groups and optochemical genetics), the boundaries between them have become vague. Here we give an account of developments over the last six years without the claim of comprehensiveness (the focus of the applications instead lies on biological systems). For the time preceding this period we refer to previous reviews.[1] Several reviews with selected foci have also appeared in the last six years.[2–12]

2. Irreversible Photo(de)activation (Uncaging)

2.1. Concepts

2.1.1. Caging Groups

One method for the regulation of molecular processes with light is the use of photolabile "protecting" groups in key locations. Ideally, this modification blocks the activity of any molecule completely and restores it only with light. This strategy is inherently irreversible. First realizations of this principle date back to 1978 when Hoffman and co-workers synthesized a photoactivatable adenosine 5′-triphosphate (ATP) and used it to study the Na/K pump.[13] Shortly before this study, Engels and Schlaeger synthesized a photoactivatable cyclic 3′,5′-phosphate of adenosine (cAMP).[14] Hoffman coined the term "caged" for biologically active compounds which have been inactivated with a photolabile group (the "cage" or "caging group"). The new concept allowed experiments to be performed which would otherwise have been difficult or impossible to do. The term "caging" is not unproblematic, because it is often misinterpreted and the impression is given that the molecule is inside an actual cage which is opened by light. Furthermore, it makes literature searches very complex: Text searches yield far too many results, since the word "caged" appears in many more contexts. The same is true for structural searches for the photolabile groups, which appear in many more contexts as well. In addition, one is prone to miss new developments if one only searches for the known scaffolds. Additionally, the unease with the term "caged" led to a diversity of alternatives, such as "photoactivatable", "light-triggered", or even "photocaged", which in turn makes text searches even more complicated. Finally, it should also be noted that there is a subtle difference between the term "uncaged" (which means after irradiation of a caged molecule) and "not caged" (which means the "wildtype" molecule itself). Ideally, both terms should mean the same, but it is important in reports on experiments to differentiate between them, because the latter is then the positive control for the former.

The requirements for good caging groups are quite complex: They should be easy to introduce as well as highly soluble and stable under physiological (sometimes intra-

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cellular) conditions. They should have a large molar extinction coefficient ε for an efficient photoactivation. However, it is not enough to only describe how well the activating light is taken up (avoiding unnecessary high doses of light), but it must also be taken into account what percentage of the molecules in the excited state undergo the desired cleavage (uncaging) reaction. This is described with the uncaging quantum yield Φ. The uncaging reaction should be fast and follow only the desired reaction pathway (the primary photoreaction is usually complete within picoseconds). To avoid photodamage the uncaging must happen at wavelengths at which the sample is transparent or where no other unintended reactions are triggered. The caging group should also be compatible with a variety of leaving groups. Since uncaging always produces a by-product (the cleavage product of the caging group), it is important that this by-product is not toxic. Also, these by-products should not absorb the light used for uncaging (better than the caged compound). It might be advantageous if the uncaging event could be monitored, for example, by the emergence of fluorescence. Finally, delivery of the caged compound is sometimes an issue, and the cage can have both positive and negative effects. Fortunately, many answers to these challenges have been found, and the literature of the last 30 years is full of successful applications of the (un)caging principle. Many excellent reviews already exist (see above). For the last six years alone we have found about 130 papers on uncaging mechanisms or new and improved versions of caging groups. It is unrealistic to give a full account of this research here. Instead, we want to focus in the following sections on two particular areas which we believe to be very interesting and conceptually important for the future of caging concepts: two-photon and wavelength-selective uncaging. Figure 1 gives an overview of the caging groups on which we will focus in the following sections, and Table 1 summarizes typical photophysical properties.

2.1.2. *Two-Photon Uncaging*

A very interesting alternative to normal "single-photon excitation" of a caging group is the two-photon uncaging strategy, where light of approximately twice the wavelength is applied.[17,18] For a caging group with an absorption maximum of 365 nm, one can try to use light of 730 nm. However, it is a true nonlinear optical effect which only happens at high light intensities. Special laser light sources have to be used—for example, a pulsed titanium sapphire laser. Typical irradiation conditions are pulses with a width of 100 fs at a repetition rate of 80 MHz and with an average power of 10–25 mW. However, even at 10 mW this still corresponds to a power of 1250 W for each ultrashort pulse. The advantage is

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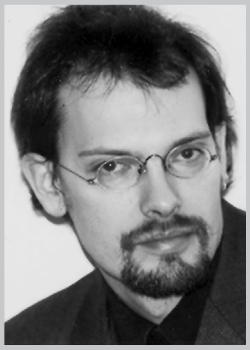
Falk Rohrbach studied food chemistry at the Technical University of Kaiserslautern and the University of Murcia. For his diploma he developed a method for the quantification of biomarkers for oxidative stress at the Novartis Institute of Biomedical Research in Basel. He joined the group of Prof. Dr. Mayer in 2010 for his PhD, and is currently applying chemical modifications to aptamer approaches for the regulation of blood coagulation and gene expression at the University of Bonn.

Alexander Gottschalk studied chemistry in Frankfurt and Marburg and completed his PhD, with Prof. Dr. Lührmann, on yeast RNA–protein complexes that mediate pre-mRNA splicing from the Philipps-University. He then studied neuronal function in the nematode C. elegans, with Prof. Dr. Schafer (UC San Diego), analyzing nicotinic receptor associated proteins, before in 2003 becoming Junior Professor at the Goethe University (Frankfurt). Together with Bamberg and Nagel, he introduced channelrhodopsin into C. elegans to allow, for the first time, light-control of its behavior. He became Heisenberg Professor in 2010.



Günter Mayer studied chemistry at the Ludwig-Maximilians University Munich. After his diploma, he obtained his PhD from the University of Bonn. From 2001 to 2004 he worked at a biotech company, heading its combinatorial biotechnology department. He obtained his Habilitation in Chemical Biology and Biomolecular Medicine in 2009, and from 2009 to 2010 was Reader for Translational Biology at the University of Strathclyde, Glasgow. Since 2010 he has been Professor of Chemical Biology and Chemical Genetics at the LIMES Institute of the University of Bonn.

Alexander Heckel studied chemistry in Constance and received his PhD from the ETH Zurich with Prof. Dr. Seebach. He then carried out postdoctoral research with Prof. Dr. Dervan (Caltech) and entered the field of DNA recognition. With a Liebig and an Emmy-Noether Scholarship, he then started his independent career at the University of Bonn, with Prof. Dr. Famulok as mentor, during which time he became interested in the regulation of oligonucleotides with light. In 2007 he became professor at the Cluster of Excellence Macromolecular Complexes at the University of Frankfurt.

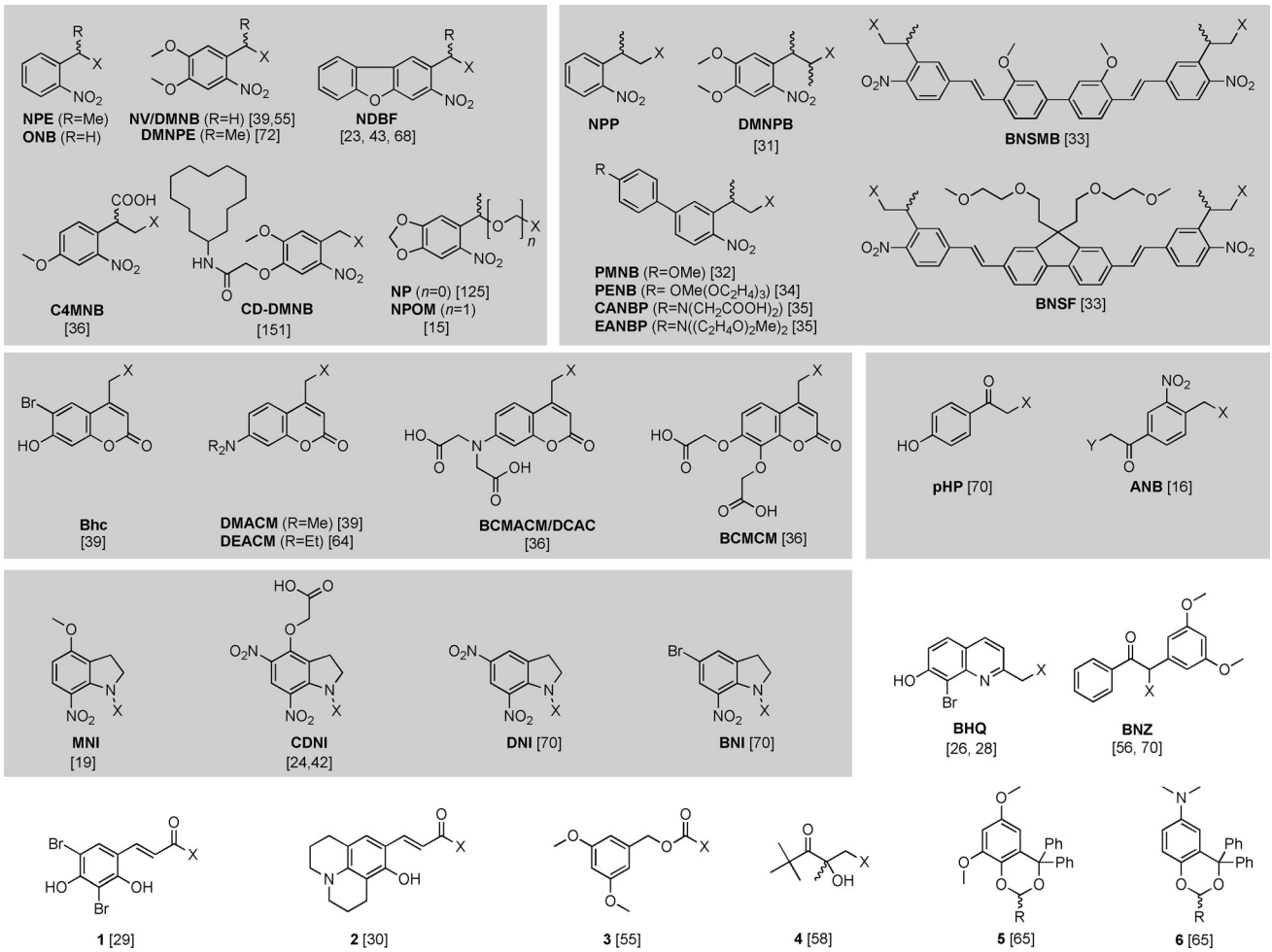


Figure 1. Overview of different caging groups. X denotes the compounds (mainly connected through alcohols, amines, or carboxylic acids) which are liberated upon irradiation with light. The references given are by no means an exhaustive account of their use. The caging groups are grouped in classes.

that the probability of a two-photon excitation is proportional to the square of the local intensity. Therefore, it is possible to adjust the irradiation conditions in such a way that excitation is only obtained at the very focus of the laser beam—thus adding a third dimension to the spatial resolution of the photolysis area (see Figure 2 left and middle). The volume in which the excitation conditions are met can be as little as 1 fL and below (see for example Ref. [19]). In combination with a suitable optical setup, such irradiation conditions made it, for example, possible to generate arbitrarily shaped objects by light-induced polymerization (Figure 2 right). Two-photon irradiation can also be used for two-photon imaging, where fluorophores are excited but no photoreaction is intended. The advantages are that the infrared light can penetrate deeper into the tissue. Also, as a result of the aforementioned three-dimensional excitation characteristics, one can get optical sections without using a confocal pinhole. Both types of application have been excellently reviewed recently.[8,20,21]

However, by no means all chromophores are well-suited to two-photon excitation. Similar to using the extinction coefficient ε and especially the product $\varepsilon\Phi$ of the extinction coefficient with the quantum yield for the desired reaction (see above), the two-photon effect can be quantified using the two-photon cross-section δ_a and the two-photon action cross-section $\delta_u = \delta_a \Phi$. The unit here is the Goeppert-Mayer (GM),

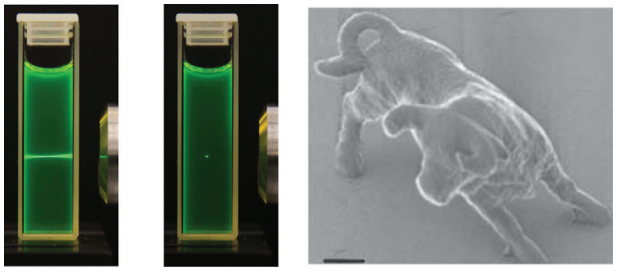


Figure 2. With two-photon irradiation it is possible to obtain three-dimensionally resolved excitation spots (middle), whereas with normal irradiation the excitation results in a double cone (left): Here, a fluorescein solution is penetrated by a laser beam of 365 nm (left) or 730 nm (middle). Only at the focus of the 730 nm beam is the intensity high enough for a nonlinear optical effect. Right: By using two-photon excitation techniques it was, for example, possible to polymerize this figurine of a bull (scale bar 2 μm, picture taken from Ref. [18]).

Table 1: Examples for optical parameters of caging groups.[a]

Caging group	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$] (λ [nm])	Φ	$\varepsilon\Phi$ [$M^{-1}cm^{-1}$]	δ_u [GM]	Ref.
NPE				240		[22]
NDBF		18400 (330)	0.7	12880	0.6	[23]
MNI			0.085		0.06	[19, 22]
CDNI		6400 (330)	0.6	3840		[24]
NV		5000 (350)	0.12	600	0.04	[22, 25]
Bhc		14800 (365)	0.036		0.72	[26, 27]
BHQ		2580 (365)	0.29		0.59	[26, 28]
1		25000 (369)	0.05	1250	1.6	[29]
2	370	20000 (394)	0.03[b]	400	4.7[a]	[30]
DMNPB	350	4500 (350)	0.26		0.17	[31]
PMNB	317	9900 (317)	0.1	990	0.45	[32]
BNSMB	400	39349 (400)	0.3	11800	0.9	[33]
BNSF	415	63960 (415)	0.25	16000	5.0	[33]
PENB			0.1	1000	3.7	[32, 34]
CANBP	397	7500 (397)	0.15	1100	7.4	[35]
EANBP	397	7500 (397)	0.15	1100	11	[35]
BCMACM	383	18500 (383)	0.06			[36]
BCMCM	324	11000 (324)	0.06			[36]

[a] The molar extinction coefficients ε as well as the uncaging quantum yields Φ and the two-photon action cross-section δ_u are given (see Section 2.1.2). For abbreviations of the caging groups, see Figure 1. It must be noted that such a comparison has to be taken with caution, since these parameters vary significantly with different leaving groups X or substituents R, and the measuring conditions were not always the same. Therefore, this Table is meant as a rough guide. [b] For primary *E*/*Z* isomerization.

which corresponds to 10^{-50} cm^4 s/photon. Some authors state that a two-photon action cross-section of >0.1 GM is desirable for biological applications.[37] An **NV** group has a two-photon action cross-section of 0.035 GM,[22] while the **NPE** and **NPP** groups have lower values (for an overview of photophysical properties see Table 1, for the abbreviations see Figure 1). Compounds with low δ_u values—such as **MNI**-glutamate with 0.06 GM—can still be used for two-photon uncaging (see, for example, Ref. [38]), and there are even reports of using **NV**-caged estrogen receptor ligands with only 0.004 GM.[39] Similarly, **NV**-caged retinoic acid with 0.025 GM has been successfully uncaged inside zebrafish embryos.[40] Interestingly, a study exists in which the two-photon action cross-section of **NV** has been estimated to be 0.23 GM. In this study, an amino acid building block was introduced, which upon **NV** uncaging led to a peptide scission by an intramolecular cyclization.[41] The authors hypothesize that this unusually high value is due to the high efficiency of the ensuing intramolecular reaction. With the exception of this single success, systematic studies have provided better two-photon caging groups in recent years.

In a series of studies, Goeldner and co-workers succeeded in improving the two-photon properties of the **NPP** group significantly: For example, the derived **DMNPB** group (for 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl) already showed a δ_u value of 0.17 GM upon uncaging glutamate.[31] In a further extension of the **NPP** chromophore, they then introduced the **PMNB** caging group (3-(2-propyl)-4′-methoxy-4-nitrobiphenyl) and found that it can be used for the uncaging of glutamate with an improved 0.45 GM.[32] Following the hypothesis that further extension of the chromophore and arrangement of donor/acceptor substituents in a dipolar or quadrupolar fashion on a linear chromophore should improve the nonlinear properties, they then created the "dimeric" caging groups **BNSMB** (4,4′-bis-{8-[4-nitro-3-(2-propyl)-styryl]}-3,3′-dimethoxybiphenyl) and **BNSF** ((2,7-bis-{4-nitro-8-[3-(2-propyl)-styryl]}-9,9-bis-[1-(3,6-dioxaheptyl)]-fluorene). Not only did those cages show the expected red-shifted absorption, but they also had very promising two-photon action cross-sections of 0.9 GM and 5 GM, respectively.[33] Recently, Goeldner and co-workers presented the **PENB** group (3-(2-propyl)-4′-tris-ethoxy(methoxy)-4-nitrobiphenyl).[34] When this **PENB** group was attached to a far-red-emitting acridinone fluorophore for cellular imaging they obtained a δ_u value of 3.7 GM. In a very recent study Goeldner, Specht, and co-workers presented the best two-photon caging groups yet: the **CANBP** and the **EANBP** groups. The latter had a remarkable δ_u value of 11 GM![35]

In an attempt to improve the two-photon properties of **MNI**, Ellis-Davies et al. came up with the **CDNI** caging group and prepared **CDNI**-caged glutamate[42] and GABA.[24] The former has a five times higher uncaging yield (determined by one-photon photolysis) than **MNI**-glutamate but explicit data on the two-photon action cross-section is not given. Ellis-Davies et al. also presented a promising new derivative of the **NPE** caging group which they abbreviate as **NDBF** (for nitrodibenzofuran). In their first study, they demonstrated its use in the release of the secondary messenger Ca^{2+} and found a two-photon action cross-section of 0.6 GM.[23] In a later study, they applied the **NDBF** group for the caging of another second messenger—inositol-1,4,5-triphosphate (IP_3)—which can be used to liberate Ca^{2+} ions indirectly from intracellular stores with a much lower average irradiation energy than previous caged derivatives.[43]

The **BHQ** group was introduced by Dore and co-workers. It can have two-photon action cross-sections as high as 0.4–0.9 GM.[28] A systematic study on the influence of substituents nicely showed once more how difficult it is to optimize the photophysical properties because in certain cases parameters could be optimized, but always at the expense of other important parameters.[44]

An interesting concept for improving two-photon action cross-sections comes from Pirrung and Dore: They used the sensitizer thioxanthone with a two-photon cross-section of 5 GM for the excitation, after which the energy was transferred to an **NPP** group.[45] Intramolecular-sensitized uncaging, albeit not by two-photon irradiation, had been demonstrated before by Pfleiderer, Steiner, and co-workers[46,47] as well as Corrie and co-workers.[48,49] In the study by Pirrung and Dore the sensitizer was not covalently linked to the caging group. As a result, they obtained a two-photon action cross-section of 0.86 GM, which is significantly higher than the one obtained with an **NPP** group alone. In a similar type of study, Li and co-workers were able to cleave an **NPE** group off a coumarin derivative with a δ_u value of 0.4–1 GM.[50,51] In this case, the coumarin was the molecule to be released for cellular imaging applications. Apparently, an interaction between the coumarin part of the molecule and the **NPE** group increased the uncaging performance of **NPE** by one

order of magnitude. However, in another study, a combination of the **DMNPB** caging group with a coumarin derivative led to only 0.21 GM.[52]

Jullien and co-workers used two-photon uncaging to control protein activity in cultured cells and zebrafish. They used the **NV** group as well as the coumarin caging groups **Bhc** and **DEACM** for the modification of 4-hydroxycyclofen, a photostable derivative of the estrogen receptor ligand 4-hydroxytamoxifen.[39] In another study, Jullien and co-workers investigated the use of the 3,5-dibromo-2,4-dihydroxycinnamic caging group (**1**), which can liberate alcohols and amines from their esters and amides.[29] Upon irradiation, the double bond is isomerized and an intramolecular attack leads to a (fluorescent) coumarin derivative, thereby liberating the caged compound with a δ_u value of 1.6 GM. In a following study, they successfully optimized the caging group and obtain two-photon action cross-sections of up to 4.7 GM for the primary *E*/*Z* isomerization of compound **2**.[30]

In a very recent study, del Campo and co-workers used a $[Ru(bpy)_2(PMe_3)]$ complex as a caging group for an aminosilane, which was used for surface patterning. The amino group could be recovered upon irradiation at 460 nm or 900 nm, but no explicit values for the two-photon action cross-section are given (a δ_u value of 0.14 GM had been determined for similar complexes in other studies).[53]

Thus, the past few years have seen a fascinating series of developments leading to improved caging groups for two-photon uncaging. "Design principles" or guidelines for improved nonlinear optical behavior have also been discussed.[8,21,33] However, while it is already difficult enough to accurately predict absorption properties, it is much more difficult to predict reaction pathways and hence uncaging quantum yields. It has to be kept in mind that the energy in the system can be dissipated in many more ways than just the intended reaction. While some of the quoted studies or others suggest rationales, very often (nonlinear) optical behavior remains a black box and some studies explicitly show how difficult it is to optimize photophysical behavior.[54] Therefore, this field is still far from being mature and much will most likely be learned in the coming years.

2.1.3. *Wavelength-Selective Uncaging*

Another interesting twist to the simple principle of uncaging and a relatively recent trend will now be discussed: the caging principle is a powerful way of obtaining light-inducible systems and it is relatively easy to obtain excellent "binary" ON/OFF behaviors. However, uncaging remains inherently irreversible. Light-induction scenarios could be designed in much more complex ways with this caging approach if it were possible to have, in one system, several caging groups which could be addressed individually with light of different wavelengths.

Pioneering work comes from the research group of Bochet, who initially studied the uncaging of esters of the caging group **3** versus **NV**-carbamates and found rate-constant ratios of 30:1 at 254 nm and 1:100 at 350 nm when comparing samples with only one of the two compounds.[55] However, this ratio could not be maintained when both compounds were present in the same solution. This cross-talk phenomenon was, however, only observed with one pair of caging groups. Probing at wavelengths from 254 nm to 419 nm and changing the substituents on the *o*-nitrobenzyl caging groups alone only afforded very modest rate-constant ratios. Thus, Bochet set up the rules that for orthogonal uncaging: a) the intrinsic stability of each protecting group should be very different at various wavelengths, b) the energy transfer between an excited chromophore and its ground-state neighbur should be suppressed, and c) the cleavage at high energy (for example, at 254 nm) should be very fast, to avoid photodegradation of otherwise sensitive groups.[56] Indeed, mixtures of an **NV**-ester and an ester of the benzoin-type **BNZ** caging group gave selectivities of 90:10 (at 254 nm) and 15:85 (at 420 nm) in acetonitrile solution.[56] It was even possible to selectively deprotect those two caging groups if they were present in the same molecule. The term "chromatic orthogonality", in analogy to "chemical orthogonality", was suggested.[57] Giese, Bochet, and co-workers then found that the **NV** group and the *tert*-butyl ketone derivative **4** were another selectively photocleavable pair, and applied them in a solid-phase peptide synthesis, with compound **4** as part of the linker.[58] **NV** served as the transient protecting group, which was removed at 360 nm, while the linker was cleaved at 305 nm. In this way, the model pentapeptide Leu-enkephalin could be obtained under essentially neutral reaction conditions. The kinetic isotope effect allowed another fine-tuning: the photolysis of *o*-nitrobenzyl cages happens through γ abstraction of the benzylic hydrogen atom by the triplet-excited nitro group. Thus, *o*-nitrobenzyl caging esters with two benzylic deuterium atoms stayed intact upon photolysis at 420 nm in the presence of undeuterated analogues (selectivities up to 8:1) because of the increased stability of the C–D bond over the C–H bond.[59] This effect could be improved by desymmetrization with different substituents on the two *o*-nitrobenzyl cages.[60] The results up to this point have also been summarized in a greater level of detail in two review articles.[61,62]

The **NV**/**BNZ** pair was adopted by del Campo, Jonas et al. for the photopatterning of glass and quartz surfaces by using orthogonal photosensitive silanes.[63] Later del Campo and co-workers established the **NV**/**DEACM** pair for sequential cleavage—first of the **DEACM** at 412 nm and then of the remaining **NV** at 345 nm—again for photopatterning.[64] In this pair, both cages are cleaved at 345 nm, but this is a nice example showing that it can sometimes be sufficient to have sequential addressability. Another pair for sequential removal was investigated by Wang et al., who used cages **5** and **6** for carbonyl groups. Irradiation with light of ≥ 350 nm led to a clean photocleavage of the latter, while both could be cleaved simultaneously with a Pyrex-filtered Hg lamp.[65]

In a very interesting study, Hagen and co-workers succeeded in the wavelength-selective uncaging of peptide thiol groups in water. This was possible after the introduction of the new coumarin cages **BCMACM** and **BCMCM** as well as the **NPE** derivative **C4MNB** with improved water solubility.[36] The two new coumarin-derived caging groups were connected through an oxycarbonyl linker to the thiol. While the **BCMACM** group still showed a significant absorption at

450 nm, the absorption of the **BCMCM** group was already very low at 360 nm. For example, with a combination of **C4MNB** and **BCMACM** in the same tetradecapeptide, the latter caging group could be released upon irradiation with $\geq$ 430 nm and then the former with light of $\geq$ 325 nm.

Ellis-Davies and co-workers combined the two-photon uncaging approach with the wavelength-selective uncaging approach and came up with two-color, two-photon uncaging by using **CDNI**-caged glutamate and **BCMACM**-caged γ-aminobutyric acid (GABA) in rat-brain slices.[66] The **CDNI** cage could be photolyzed at 720 nm, while the **BCMACM** cage was cleaved at 830 nm. In this way, two caged neurotransmitters could be orthogonally activated with control over the space, time, and dose. In a similar study Lawrence and coworkers used **BCMACM**-caged 8-bromo-cAMP in the presence of the *o*-nitrobenzyl-caged protein kinase PKG (caged on a cysteine residue). Either the coumarin cage was photolyzed selectively at 440 nm or both together at 360 nm.[67] Thus, they could wavelength-selectively address two different nodes of a common signaling pathway.

In our own contribution to this field we have extended the principle of wavelength-selective uncaging to nucleic acids and shown that it is possible to photolyze **NDBF**-caged deoxyadenosine or -cytidine residues as well as **DEACM**-caged deoxyguanosine residues in the presence of their **NPE**- or **NPP**-caged counterparts at $>$ 400 nm.[68,69] Selectivities of up to 80:1 were obtained in this example of sequential uncaging.

San, Bochet, and del Campo recently asked the question how many functional levels are possible in wavelength-selective uncaging.[70] By using the caging groups **NV**, **NPP**, **BNZ**, **DEACM**, **DNI**, **BNI**, and **pHP** on silanes terminated with amines, thiols, and carboxylic acids for the modification of surfaces they demonstrated that up to four different and independently addressable functional levels can be realized with the current technology. However, wavelengths below 300 nm were used in this study, and it is questionable as to how applicable this would be in a biological system. While this variety is already quite extraordinary, it is certainly correct to say that wavelength-selective uncaging is still an emerging field that has only recently come to fruition in its application to surfaces and biomolecules, and again it will be exciting to see the progress over the coming years.

2.2. Caged Small Molecules

2.2.1. Compounds for Gene Expression

Regulation of gene expression is an important task in several research fields, for example in molecular systems biology, and caged compounds give access to a further level of control over these processes, now with spatiotemporal resolution. Methods towards small-molecule-regulated gene expression are currently available for almost every cell type. Approaches to control these systems by using caged compounds have also been accomplished in several ways. For example, Young and Deiters employed nitropiperonal to generate a caged version of isopropyl-β-D-thiogalactoside (IPTG; Figure 3), which releases the lactose repressor from

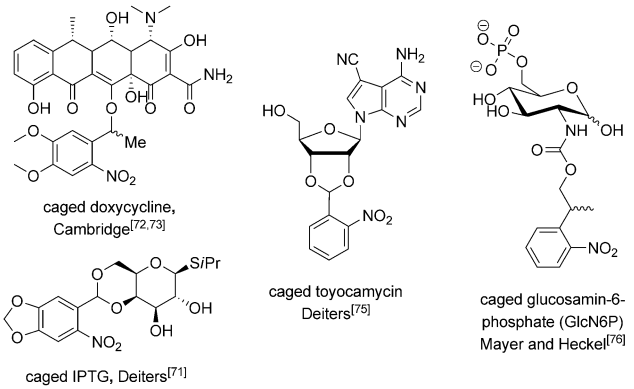


Figure 3. Overview of caged compounds used for the regulation of gene expression with light.

the lac operator and enables gene transcription (Figure 3). Consequently, the incorporation of this compound into *Escherichia coli* enabled the light-induced expression of a reporter gene.[71]

Cambridge et al. used the famous tetracycline-on (Tet-on) system to develop photoactivated gene expression in eukaryotes and synthesized a **DMNPE**-caged version of doxycycline (Figure 3). When applied in Chinese hamster ovary, mouse embryos, and *Xenopus laevis* tadpoles, it could induce green fluorescent protein (GFP) expression with single-cell resolution by the two-photon uncaging of doxycycline.[72,73] Koh and co-workers made use of a similar approach involving **NV**-caged doxycycline to express ephrin A5 in NIH 3T3 cells, thereby directing the arrangement of cells on living cell monolayers (Figure 3).[74]

In addition to small-molecule protein effectors, compounds that regulate the activity of ribozymes were also investigated to achieve photoactivatable gene expression. Ribozymes represent self-cleaving RNA elements, and when embedded in mRNA molecules they can be employed to control gene expression. Deiters and co-workers synthesized an **ONB**-dioxolane-caged version of toyocamycin, which is an inhibitor of the hammerhead ribozyme (Figure 3).[75] Uncaging of toyocamycin in mammalian HEK 293T cells that were transfected with a plasmid encoding for the reported gene luciferase under control of a hammerhead ribozyme resulted in an increase in the luciferase activity. In our own study we used the *glmS* ribozyme to control GFP translation in reticulocyte lysates and could control the GFP production with light by using an **NPP**-caged version of the ribozyme's cofactor glucosamin-6-phophate.[76] Currently, this approach is limited to cell-free systems, since the highly polar cofactor cannot pass through the lipid bilayer of eukaryotic cells.

2.2.2. Compounds in Neurobiology and Signaling Pathways

Neurons communicate through synapses by using the amino acid glutamate and other effector molecules. Glutamate in particular has been equipped with different photolabile groups. The commercialization of **MNI**-glutamate allowed various investigations with two-photon uncaging in neuroscience, even for scientists without a synthesis labora-

tory.[77–83] It is noteworthy that **MNI**-glutamate has been employed in vivo, and demonstrated spatiotemporal structure–function relationships of dendritic spines localized in the neocortex.[38] Single spines could be engaged with glutamate with a spatial resolution of uncaging of 0.8 μm and led to a transient current in the living mouse brain.

Second-generation nitroindolinyl-caged neurotransmitters, namely **CDNI**-Glu and **CDNI**-GABA, have been shown to be more effective at firing and blocking the action potential in hippocampal neurons. Ellis-Davies et al. applied both caged neurotransmitters and two-photon uncaging in living rat brain slices at axial resolutions of about 2 μm, thereby allowing high-resolution functional mapping of GABA-A and AMPA receptors.[24,42,84,85] The combination of **CDNI**-Glu, uncaged at 720 nm, with **DCAC**-GABA, uncaged at 830 nm, enabled the multimodular optical control of the membrane potential of a single synapse.[66] Recently, Goeldner and co-workers synthesized new photoactivatable derivatives of GABA-**CANBP** and **EANBP**-GABA, which can be uncaged even at 800 nm and were applied for the release of GABA in brain slices.[35]

Vanilloid derivatives can induce nociceptive neuron membrane depolarization through heat-sensitive transduction channels. Different caged ligands of the vanilloid receptor were synthesized to investigate these events with spatiotemporal resolution. Kao and co-workers demonstrated extracellular receptor activation by two-photon uncaging (720 nm) of the **NV** variant.[86] In similar experiments, Frings and co-workers demonstrated reduced intracellular receptor sensitivity by one-photon uncaging of the **BCMACM** variant.[87]

Cyclic nucleotides are secondary messengers that are involved in many different signal-transduction pathways and, thus, have been intensively studied. Caged versions have been commercially available for more than a decade and have paved the way for the application of caged cGMP, in particular, as a standard for signaling investigations. Recently, Kaupp and co-workers utilized the activation of **DEACM**-caged cGMP to induce voltage responses in cyclic-nucleotide-gated channels in the sperm flagellum of marine invertebrates. This approach afforded ground-breaking insights into the chemoattractant signaling by the application of a caged compound.[88]

Phosphoinositols (IPs) are important components of the intracellular transduction network and exhibit various phosphorylation patterns. An **NPE**-caged version of 1,4,5-IP_3 is commercially available, and **NV**-caged IP_4 was employed for more than a decade for the investigation of intracellular signaling. These phosphoinositol derivatives are rendered membrane-permeant by bioactivatable protecting groups. In recent years, Ellis-Davies and co-workers developed two new caged versions of 1,4,5-IP_3, namely **NV**-IP_3 and **NDBF**-IP_3.[22,43,89] The former provided new insight into the understanding of astrocyte-synapse communication by using two-photon uncaging (720 nm).[90] **NDBF**-IP_3 has a fivefold higher quantum yield than **NV**-IP_3 and, therefore, may lead to an increased mobilization of calcium in neurons.

Schultz and co-workers synthesized **DEACM**-caged versions of phosphatidylinositol 3-phosphate PtdIns(3)P and PI(3,4,5)P_3 (Figure 4)[91,92] These compounds are membrane-permeable, similar to other caged phosphoinositides, which can be metabolized by endogenous enzymes to remove the protecting groups. Caged PtdIns(3)P was applied to induce spatiotemporal endosomal fusion in HeLa cells, and thereby demonstrated phophatidylmonophosphate to be a second messenger connecting phosphatidylinositol-3-OH kinase signaling with the endosomal pathway.

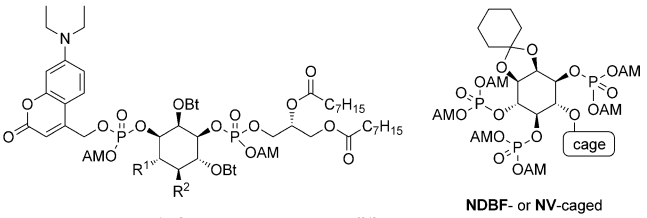


Figure 4. Overview of caged phosphoinositols for light-induced triggering of cellular signaling pathways. AM = acetoxymethyl, for cellular delivery; Bt = butyroyl.

Diacylglycerol is, in addition to IP_3, another secondary messenger that is hydrolyzed by phospholipase C. The role of diacylglycerol in the transient orientation of the microtubule-organizing center of T cells toward an antigen-presenting cell was described by Huse and co-workers, who used a **Bhc**-caged version.[93]

Calcium is the most important carrier of chemical information and regulates a wide variety of functions in cells. Caged chelating ligands that release Ca^{2+} ions upon irradiation have been used for decades, particularly in neurobiology. A comprehensive review of these ligands and their functions has been published recently.[94]

Bittman and co-workers synthesized **DEACM**- and 4-bromo-5-hydroxy-2-nitrobenzhydryl-caged versions of ceramide and ceramide-1-phosphate, which are important components of the cell membrane and also signaling molecules.[95,96] These sphingolipids were taken up by macrophages, and after uncaging an increased cell proliferation was observed.

The rapamycin-mediated heterodimerization of the FK-506 binding protein and FKBP-rapamycin binding protein was the subject of several studies using **NV**-caged rapamycin. By photoactivation of rapamycin, Woolley and co-workers induced the time-resolved interaction of FKBP and mTORC1 and thereby inhibited mTORC1 signaling in HeLa cells.[97] Umeda et al. fused YFP and Tiam1, a specific guanine exchange factor for Rac, with FKBP.[98] The dimerization of the FK-506 binding protein and YFP-FKBP-Tiam1, triggered by the uncaging of rapamycin, enabled local Rac activation and subsequent ruffle formation in NIH 3T3 cells. Deiters and co-workers employed the heterodimerization technique in a similar approach for the activation of an engineered protein kinase by light.[99] They connected this protein kinase to an engineered version of FKBP and demonstrated the local control of membrane ruffles in HeLa cells by the uncaging of rapamycin.

2.2.3. Compounds with Hormone Function

Hormones and their receptor binding analogues regulate a variety of functions in living systems. Caged versions of several compounds in this class were generated to regulate gene expression. Recently, Jullien and co-workers developed a **DMACM**-caged version of retinoic acid, which is an important signaling molecule during embryogenesis.[40] Releasing the compound by two-photon uncaging in zebrafish embryos led to a retinoic acid induced retina malformation. The same research group also synthesized an **NV**-caged version of 4-hydroxytamoxifen, the inducer of the estrogen receptor ligand binding domain (ER^{T2}).[39] ER^{T2} was fused to GFP and inactivated by a chaperone complex. Fluorescence could be induced in the embryos by one- or two-photon uncaging of the ligand. Koh and co-workers synthesized an **NV**-caged vitamin D analogue.[100] Vitamin D receptor mediated luciferase reporter activation could be observed in HEK 293T cells upon uncaging of the ligand. **Bhc**-caged progesterone was employed to study its rapid, nongenomic effect on human sperm.[101] One- or two-photon uncaging resulted in the influx of Ca^{2+} ions and a change in the swimming behavior of the sperm cells.

2.2.4. Other Compounds

Besides the release of stable molecules from caged precursors, caged compounds are also applied for the formation of species, such as nitric oxide (NO) or hydrogen peroxide (H_2O_2), involved in oxidative processes. Miyata and co-workers reported the formation of NO by the two-photon excitation of fluorescein linked to 2,6-dimethylnitrobenzene.[102] Chang and co-workers caged 1,2,4-trihydroxybenzene, which reduces molecular oxygen to superoxide and leads to the formation of H_2O_2.[103] H_2O_2 activates cofilin, an actin depolymerization factor at the actin rods in HeLa cells. Uncaging of 1,2,4-trihydroxynitrobenzene triggered the reaction in physiologically relevant amounts and was visualized by GFP fused to cofilin.

Classical caged compounds are directly covalently bound to their photolabile group. Mokhir and co-workers developed a remarkably different system for the photorelease of the molecule of interest.[104] They fused two fluorophores, which quench each other, to 1,9-dialkoxyanthracene, a singlet oxygen ($^1O_2$) sensitive linker. Irradiation with light led to a photosensitizer that formed $^1O_2$ from triplet oxygen in vitro, which cleaved the linker and released the fluorophores. The system also was used to monitor $^1O_2$ concentrations in HeLa cells.

2.3. Photo(de)activatable Peptides and Proteins

2.3.1. Photoactivatable Peptides

Peptides are used in molecular biology mostly to activate or inhibit protein function. Cages are introduced to prevent these short amino acid sequences from binding to or performing their natural function at the target. Kinases phosphorylate their target substrates in response to specific activating signals. Bresnick, Lawrence, and co-workers employed this incidence to activate a peptide substrate for protein kinase C (PKC), which changes its fluorescence upon phosphorylation.[105] A **DMNB** cage at a specific residue of the peptide inhibited its phosphorylation. Hence, the activity of PKC in PtK2 cells during mitosis could be monitored in a time-resolved manner by uncaging.

In a very sophisticated approach, Lawrence and co-workers designed a kinase-inhibiting caging agent. This agent consisted of two parts connected through a **DMNPE**-based photocleavable linker.[106] One part of this caging agent contained a maleimide and a TAMRA fluorophore. The other part contained an inhibitory peptide, which binds to the active site of PKC, and a quencher. Thus, the bound inhibitory peptide was anchored intramolecularly through the maleimide. Irradiation of REF52 cells cleaved the construct, thus liberating the inhibitory peptide and the quencher. This restored the PKC activity and the fluorescence (as a marker for the activity).

The fibronectin epitopes RGDS (arginine-glycine-aspartate-serine), a short peptide that promotes integrin-mediated cell adhesion, were caged in different ways to obtain photoinducible cell adhesion on surfaces. Stupp and co-workers synthesized an RGDS peptide bearing a palmitoyl tail and an **ONB** cage at the N terminus.[107] This molecule self-assembles into nanospheres, but forms nanofibers upon UV irradiation. NIH/3T3 mouse embryonic fibroblasts incubated with the caged peptide exhibited an increased expression of the cytoskeletal protein vinculin, found in focal adhesion plaques, after photoreleasing, thus indicating an increased bioactivity. A similar approach was used by Del Campo and co-workers, who introduced a **DMNPB**-caged aspartate in the cyclic form of RGD, and the caged peptide was immobilized on a silica surface through a bifunctional tetra(ethylene glycol) linker.[108] Cao and co-workers fused a RGDS variant with an **ONB** cage at the glycine residue associated with a hyaluronic acid hydrogel.[109] In both cases uncaging led to an induced adhesion of fibroblasts.

Kikuchi and co-workers employed a bacterial liposome as a compound release system that could be opened by a liposome-degrading antimicrobial peptide (AMP).[110] The 13th amino acid of the AMP was caged by a **Bhc** moiety at the respective lysine residue. Carboxyfluorescein-containing giant unilamellar vesicles were shown to be efficiently degraded by the uncaging of AMP with UV light.

Kuner et al. also applied a cage at a lysine residue in a peptide that inhibits the *N*-ethylmaleimide-sensitive factor so as to investigate the timing of neurotransmitter release.[111] Microinjection of the peptide into the presynaptic terminal of the squid giant synapse and photolysis of the ((5-carboxymethoxy-2-nitrobenzyl))oxy)carbonyl cage with UV light resulted in inhibition of the formation of the SNARE complex (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor complex).

2.3.2. Photodegradable Peptides

The functionalization of a peptide with a cage group may not only result in the inhibition of the peptide function and its

reconstitution of activity after irradiation. Caged peptides can also be designed with preserved function, and photocleavage results in the loss of binding to their target (Figure 5). Kron and co-workers introduced the nitrophenyl amino acid

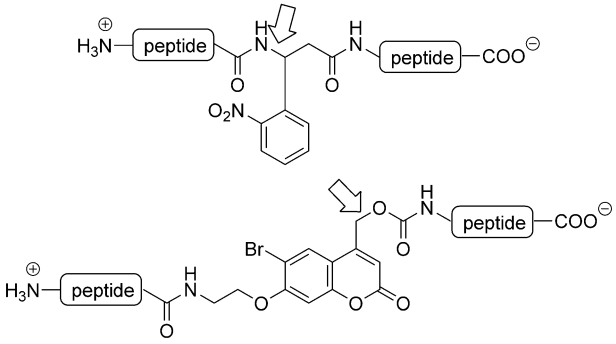


Figure 5. Two approaches to photocleavable peptides through either an **NPE**- (top)[112–115] or a **Bhc**-based strategy (bottom).[116] The photocleavable bond is marked with an arrow.

derivative (2-nitro)-β-phenylalanine in the backbone of the peptide pheromone α factor from *Saccharomyces cerevisiae*.[112] This photodegradable peptide activated the mating pathway as well as its unmodified version and thereby arrested the cell cycle in the G1 phase. Irradiation with UV light resulted in recovery of G1 arrest, as characterized by an α factor/nocodozole trap assay and flow cytometry.

Ovaa, Schumacher, and co-workers synthesized a version of a major histocompatibility complex (MHC) class I ligand containing an **ONB**-based amino acid.[113] This photocleavable nine amino acid peptide kept biotinylated MHC in its stable, peptide-bound form. UV irradiation resulted in the complex degrading and enabled the association of biological ligands of interest to the MHC. This reloading with different epitopes of choice allowed antigen-specific T-cell responses to be monitored in a high-throughput manner.

Piehler et al. synthesized a photofragmentable oligohistidine peptide for the site-specific targeting of His-tagged proteins.[114] The **ONB-**based peptide containing photocleavable amino acids blocks tris-NTA-functionalized surfaces, but the His-tag binding capacity disappears upon irradiation.

Lawrence and co-workers designed a bivalent photocleavable inhibitor of the active site and the regulatory domain (SH2) of the Src tyrosine kinase.[115] Photolysis of the **ONB** cage, which connects the two peptides, resulted in recovery of the Src kinase activity in vitro.

Aiming for higher photolytic efficiency, Nagamune and co-workers introduced the **Bhc** group as a linker in model peptide conjugates (Figure 5).[116] The photosensitivities were higher than **ONB**-caged linkers. Smith, Hochstrasser, and co-workers synthesized a cyclic peptide with a tretrazine-based linker.[117] This ultrafast chemical trigger exhibited complete photolysis after flash irradiation at 256 nm and slower photolysis up to the visible range, thus enabling the initiation of early events in peptide folding.

2.3.3. *Caged Genetically Encoded Proteins*

The development of new orthogonal aminoacyl-tRNA synthetase/tRNA pairs enabled the introduction of a variety of caged amino acids in proteins by altering the genetic code of *Escherichia coli*, yeast, and mammalian cells.[118] Applications for **ONB**-caged tyrosine, **ONB**-caged fluorotyrosine, **DMNB**-caged serine, and **NPOM**-caged lysine were recently reported.

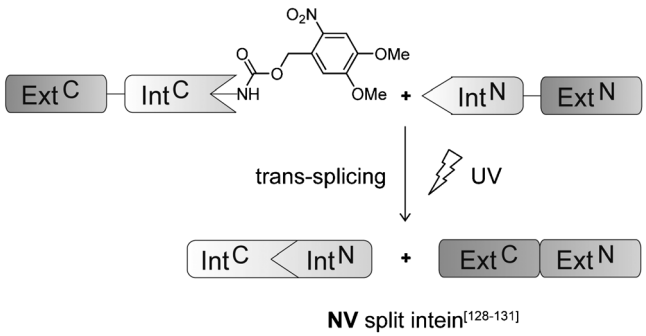
Deiters et al. genetically encoded **ONB**-caged tyrosine for incorporation into the hydrolase β-galactosidase and the DNA polymerase I of *Thermus aquaticus* in *Escherichia coli*. This led to UV-light-inducible β-galactosidase activity and DNA polymerization.[119,120] By utilizing the same expression system, the topoisomerase Cre recombinase, T7 RNA polymerase, and a zinc-finger nuclease with caged amino acid residues were generated, this time in mammalian HEK 293T cells. In this way, GFP fluorescence mediated by restored recombinase activity, T7 promoter procured gene transcription, and site-specific double-strand breaks induced by zinc finger proteases and leading to reporter gene activity could be observed.[121–123]

Furthermore, a pyrrolysyl-tRNA system was developed that encodes **NP**-caged lysine in HEK 293T cells. The tumor suppressor p53 and the mitogen-activated protein kinase 1 (MEK-1) were expressed with the photoactivatable amino acid placed in their active site. The localization of EGFP-fused p53 could be monitored upon UV irradiation, and the MEK-1 activity was measured by extracellular-signal-regulated kinase phosphorylation.[124,125]

DMNB-caged serine was introduced by Schultz and co-workers into the transcription factor Pho4 in *Saccharomyces cerevisiae*.[126] Photolysis at 405 nm resulted in Pho4 being phosphorylated by the cycline-cycline dependent kinase complex and exported from the nucleus to the cytoplasm. Not only were proteinogenic amino acids functionalized with photolabile groups: The aminoacyl-tRNA synthetase system was shown to accept further chemically modified caged amino acids. This approach was used by Deiters, Cropp, and co-workers for the site-selective introduction of a fluorotyrosine in EGFP expressed in *E. coli* to alter the electronic properties of the chromophore.[127]

2.3.4. *Photomodulation of Protein Splicing*

Protein splicing is the autocatalytic cleavage of an internal protein domain (Int) from a peptide sequence followed by ligation of its flanking regions (Ext).[128] Muir and co-workers synthesized a version of the split *Ssp* DnaE intein caged with an **NV** residue at the α-amino group of Ser35.[129] Uncaging with UV light resulted in the in vitro formation of splicing product comparable to the unmodified peptides quantified by Western blot (Scheme 1). Camarero and co-workers used a similar approach to introduce an **NV** cage in the peptide backbone of intein.[130] Photocontrollable formation of the model maltose binding protein by protein splicing was measured by a change in the fluorescence anisotropy arising from the labeling of the ^{C}intein with a fluorophore. Mootz and co-workers synthesized an intein with an **NV**-caged diamino



Scheme 1. Schematic representation of the light-triggered splicing of proteins.

propionic acid, the isostere of cysteine.[131] By applying prothrombin as the extein in this system, they could induce coagulation in human blood plasma with UV light.

2.3.5. *Other Applications*

To address fluorophores in certain proteins in a spatiotemporal manner, for example, for microscopy applications, Johnsson and co-workers fused the O^6-alkylguanine-DNA alkyltransferase (SNAP tag) to the target protein and a SNAP-tag reactive moiety to the photoactivatable fluorophore group. Photodegradation of a linker between the fluorophore and its quencher or photolysis of a directly caged fluorophore resulted in the generation of a fluorescent-labeled SNAP-tagged protein in mammalian cells and zebrafish.[132–134]

Investigating the ubiquitylation of proteins is essential for understanding cellular processes, such as proteosomal protein degradation and the regulation of gene transcription. Therefore, Muir and co-workers designed a sophisticated expressed protein ligation system, including a photolytically removable ligation auxiliary, to obtain site-specific ubiquitylation.[135,136] A C-terminal peptide of the histone H2B was protected with **ONB**-based photocleavable groups and ubiquitin was site-selectively attached by auxiliary-mediated expressed protein ligation. The protecting groups were then removed by irradiation at 365 nm, and the N-terminal part of the H2B protein was ligated to the peptide. With this chemically ubiquitylated H2B (uH2B) they could prove direct stimulation of the K79-specific methyltransferase hDOT1L through uH2B, as evident by histone H3 methylation.

In some of the approaches described above, the protein of interest is caged at an amino acid that resides in the active site and is going to be phosphorylated by its natural activator. Imperiali and co-workers chose a different approach, in which they directly introduced caged phosphorylated amino acids in peptides and proteins by solid-phase synthesis.[137] They designed **DEACM**- and **NPE**-caged versions of phosphorylated serine and applied them to the substrate and an inhibitor of the Wip1 phosphatase, which allowed wavelength-selective activation and inactivation of a phosphorylation pathway.[138] An **NPE**-caged phosphorylated serine was introduced in the myosin regulatory light chain, and spatial control over the release of activated myosin was achieved by uncaging in COS7 cells.[139]

2.4. *Photo(de)activatable Oligonucleotides*

Caged derivatives of DNA, RNA, and of their analogues became available rather late in the history of caging compared to reversibly photoswitchable DNA. For an account of these early studies we refer the reader to our previous review.[1] Understandably, the last six years have seen the development of quite a diverse range of new applications. Conceptually, in oligonucleotides, the caging group can be introduced on the nucleobases to prevent Watson–Crick base pairing, on the ribose, the phosphodiester backbone, or as an internal photocleavable linker (Figure 6). The effect of nucleobase caging on duplex stability has been

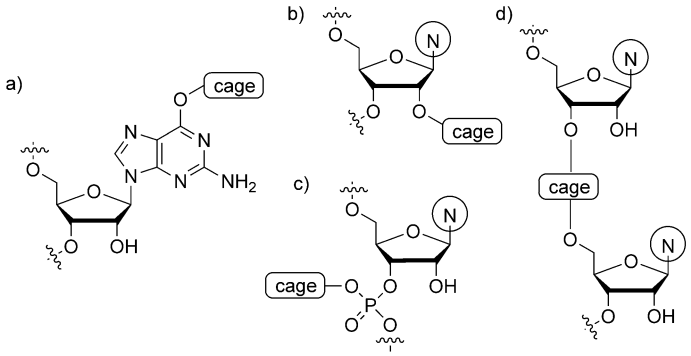


Figure 6. Overview of different caging concepts for DNA or—as here—RNA. The caging groups can block the a) Watson–Crick base paring capabilities, b) 2′-OH groups of RNA,[141] or c) residues of the phosphodiester backbone, or d) be present as photocleavable linkers between two nucleosides or on the 5′ or 3′ ends.

systematically studied.[140] A photocleavable linker can also be used on the 5′ or 3′ end. While the modifications of type (a), (b), and (d; Figure 6) are usually synthesized by using suitable precursors in a solid-phase synthesis, type (c) is realized by an (unspecific, statistical) alkylation of oligonucleotides with diazo precursors of caging groups.

2.4.1. *Regulation of Gene Expression by Light*

Most of the investigations dealt with the regulation of gene expression and has been reviewed before.[142–146] The use of RNA interference is probably one of the most interesting ways of regulating genes, where the key players are small interfering "siRNAs". They can, for example, be made to be light-inducible by caging the nucleobases in the center of the so-called guide strand, as we showed in our own study.[147] Friedman and co-workers were the first to describe light-inducible siRNAs that were caged on the phosphodiester backbone (Figure 6c);[148] however, the ON/OFF effect was incomplete. In an attempt to improve this, they modified the 5′ end of the guide strand with caging groups and again found

only an incomplete ON/OFF behavior.[149] This was in contrast to expectations from the literature, which until that time stated that any 5′ modification abolishes RNAi activity completely. In a following study on the course of the modification of oligonucleotides with diazo precursors of caging groups they found that, in contrast to usual expectations, the terminal backbone phosphate groups of siRNAs are caged much more easily than internal phosphodiesters or nucleobases.[150] To perfect their terminal-caging approach they switched to a sterically more-demanding caging group **CD-DMNPE** and indeed obtained a better light-induction behavior.[151] Since the stability of the caged constructs until photoactivation is an issue, they also showed that caged siRNAs with phosphorothioate linkages have an increased stability in serum.[152] Monroe and co-workers performed a similar study with 2′-fluoro-modified siRNAs in cell culture and in zebrafish embryos.[153] Blocking the 5′ end of the antisense strand is also the strategy followed by McMaster and co-workers. They could successfully downregulate—even in a dose-dependent fashion—a number of genes without generating nonspecific effects.[154]

A more traditional approach to the regulation of gene expression is the use of antisense oligonucleotides which either block the mRNA or induce its cleavage by RNAse H. In a series of studies Dmochowski and co-workers prepared light-activatable antisense reagents, for example, by modifying an antisense oligonucleotide against *c-myb* RNA through a photocleavable linker with a partially complementary sense strand (see Figure 7a). This intramolecular hairpin could be photocleaved, thereby liberating the antisense agent.[25,155–157] Mokhir et al. synthesized a linker that can be photolyzed with red light for such an approach. Its mode of action is based on the generation of singlet oxygen through a photosensitizer.[158] Deiters and co-workers, on the other hand, used nucleobase-caged phosphorothioate antisense agents which only hybridized to the cognate mRNA upon uncaging in mouse fibroblast cells.[159] They later showed that it is possible to deactivate antisense reagents with light by using a construct with an oligonucleotide, a photocleavable linker, and polyethylene glycol (PEG).[160] The PEG protected the DNA antisense agent from degradation by exonucleases until photocleavage. A special sort of antisense reagent is the so-called "morpholinos", in which the ribose unit has been replaced by a morpholine ring for better stability inside of cells. Chen and co-workers described light-activatable morpholinos in which the activation strategy again consisted of an antisense strand which was attached through a photocleavable linker.[161–163] They showed that this system can be used to regulate the *no tail* gene with light in zebrafish embryos. Deiters, Yoder et al. showed that it is also possible to use a nucleobase-caged approach and regulated *chordin* expression in zebrafish.[164]

"AntimirRs" are antisense oligonucleotides with reactivity against micro-RNAs (miRNAs). By using a photocleavable linker approach, Li and co-workers successfully created the first caged antimirs and successfully applied them in *C. elegans*.[165] Genes that are under the control of transcription factors can also be regulated with "decoys"—extra copies of the dsDNA transcription factor binding site. Deiters and co-workers showed that nucleobase caging can produce inactive decoys and could regulate NF-κB activation through uncaging.[166] The translation of plasmids was successfully regulated by Nagamune and co-workers through the site-specific introduction of a caged residue into the plasmid in a PCR reaction.[167] By joining two shorter antisense strands with a photocleavable linker, Dmochowski and co-workers created "RNA bandages", which presented a roadblock for ribosome translation.[168] Cleavage of the linker restored translation.

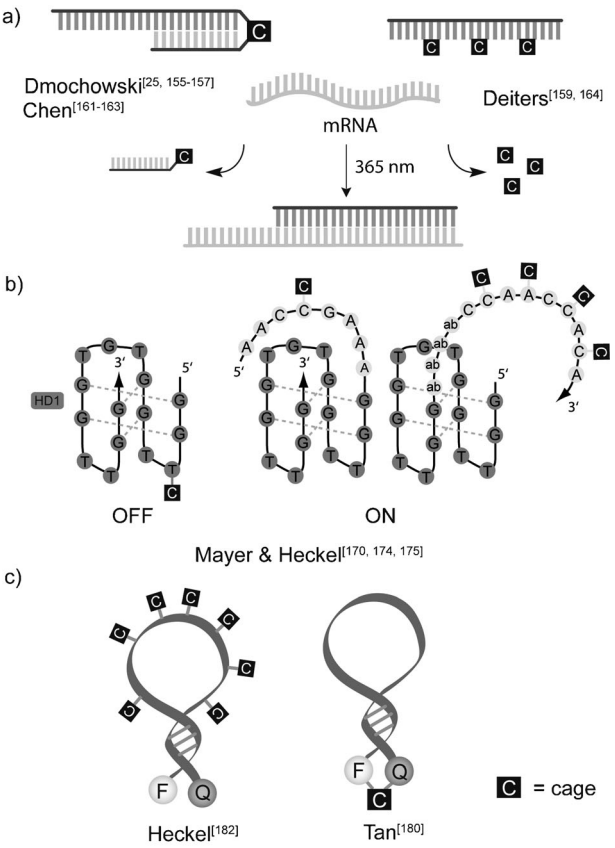


Figure 7. Some applications of photo(de)activatable oligonucleotides: a) light-regulation of gene expression with caged antisense oligonucleotides; b) regulation of aptamer activity; and c) caged molecular beacons.

2.4.2. *Regulation of Aptamers by Light*

Another very valuable application of oligonucleotides is their use as aptamers, for example, for the regulation of protein function.[169] Aptamers are obtained in an evolutionary selection process termed SELEX, in which a target of interest is incubated with a large library of DNA or RNA. Without any further necessary knowledge on the interaction properties of the target binding, aptamers are identified in several cycles of washing away unbound material and PCR amplification of what has bound. Aptamers can also be used for diagnostic purposes and much more. Hence, it is desirable to control their function with light. We reported the first light-activatable aptamer:[170] We showed that the activity of the aptamer **HD1** which forms a G-quadruplex structure and can

inhibit the serin protease thrombin can be triggered with light by caging one single thymine nucleobase involved in the interaction (Figure 7b). Molecular dynamics simulations showed the influence of the caging group.[171] Guo, Shi, and co-workers later showed that backbone caging is also applicable with the same aptamer.[172,173] Since this aptamer is of therapeutic interest as a blood-clotting inhibitor, we then showed that it is also possible to turn the aptamer activity off with light by using an intramolecular antisense ("antidote") strategy.[174] Once liberated with light, the base-pairing capabilities of the antisense strand destroy the active G-quadruplex formation of the aptamer. It turned out that it was very important whether this antisense strand was attached to the 5′ or 3′ end and the latter even resulted in more potent blood-clotting inhibitors—an effect which is not fully understood.[175] While it was convenient to have an X-ray structure of **HD1** and thrombin, this is not necessary for finding good caging sites. Simple predictions of the secondary structure can be sufficient if one decides to prevent the formation of the active conformation of the aptamer with caged nucleobases.[176] Since aptamers can even be generated to target different regulatory sites on proteins, we also showed that domain-specific light-regulation of protein function is possible by using bivalent aptamers with cages in the different contributing aptamer halves.[177] In a recent study we showed that caged aptamers can also be used for the functional detection of proteins in a process called apta-PCR, in which a diagnostic aptamer is liberated from its target so as to be quantified and analyzed by quantitative PCR.[178]

2.4.3. *Other Applications*

A very recent development are caged molecular beacons.[179] Molecular beacons are diagnostic oligonucleotides which form a stem-loop structure and have a fluorophore on one end and a quencher on the other. In this state, the fluorescence resonant energy transfer (FRET) effect between the two makes the beacon nonfluorescent. However, upon binding of the loop region to a target mRNA, the stem-loop structure opens and the beacon fluoresces—thus, indicating the presence of the cognate RNA. Caged molecular beacons require an additional light-trigger for this process. The objective is that they can be used for single-molecule real-time detection of RNA in living cells with minimal background because they can be activated for RNA probing only where desired by a focused laser. Two approaches exist for this up to now (Figure 7): Tan and co-workers showed that such an effect can be realized by connecting the two ends of a molecular beacon through a photocleavable linker.[180] In our study we demonstrated that the effect can be improved to an almost binary ON/OFF behavior if the nucleobases in the loop region are caged with multiple cages which could still be removed efficiently.[181] Abe, Ito, and co-workers showed that a similar effect is possible when using an oligonucleotide with a caged fluorophore.[182] However, here the probe also lights up in the absence of the target RNA.

One of the many next-generation sequencing technologies is the sequencing by synthesis approach.[183] This is basically a primer extension technology, in which reversible terminators ensure that only one (fluorescent) residue is attached in every cycle. Which base it is can be detected by the incorporated color. The reversible terminator and the fluorophore are then removed and the next cycle begins. Of course, uncaging can be elegantly used here. A report by Metzker and co-workers shows that nucleobase caging of the exocyclic amino group of adenosine alone already results in an effective reversible termination of primer extension.[184] The triphosphate is recognized by several polymerases, but further elongation is only possible after uncaging. In another study, they introduced **ONB**-caged 5′-hydroxymethyl-2′-deoxyuridine triphosphate (**HOMedUTP**).[185] This thymidine analogue also behaved as a photocleavable 3′-OH-unblocked reversible terminator. A recent study completed the final set of caged reversible terminators.[186]

In a series of studies Komiyama, Kuzuya, and co-workers applied the concept of nucleobase-caged nucleotides to PCR reactions. For example, by using a caged $\mathbf{dT^{NPP}}$ in a DNA template they were able to stop the primer extension of the counterstrand at the position opposite the caged residue. Thus, after photolysis they were able to prepare double strands with sticky ends which could immediately be used for ligation into a plasmid vector.[187–190] Deiters and co-workers expanded the scope of this application and showed that it is possible to elegantly perform site-directed mutagenesis or deletions in plasmid DNA.[191] They also controlled PCR reactions with light in a different way: They used primers with three cages—thereby preventing annealing to the template—to activate primer hybridization with light. Deactivation of PCR reactions was possible by using primers with caged antisense regions which could form hairpin structures upon irradiation with light and thus dehybridize from the template upon irradiation.[192]

Deiters and co-workers also showed that ribozyme activity can be brought under the control of light: ribozymes are catalytically active RNA that can, for example, catalyze the cleavage of a phosphodiester bond. They can be engineered for substrate selectivity and conditional activity, for example, sensing the presence of small molecules. Theophylline is such a small molecule which can control an engineered variant of the hammerhead ribozyme. Young and Deiters prepared caged versions of theophylline as the light-activated ribozyme.[193] While this study could have been mentioned in Section 2.2 on caged small molecules, the authors then proceeded to show that it is also possible to introduce nucleobase-caged residues into two DNAzymes (made from DNA instead of RNA) and control their function with light.[194,195] By using an antisense approach it was also possible to also photoregulate DNAzymes.[196] Here, a caged DNA analogue (decoy) of the RNA to be cleaved was used. Once activated with light, it binds to the DNAzyme but is not a substrate for cleavage. This caged antisense strand could also be present as an intramolecular extension of the DNAzyme. In a similar approach, Dmochowski and co-workers turned the 10–23 DNAzyme on or off with light by using a photocleavable linker residue in the DNAzyme which either cleaved and hence deactivated the DNAzyme or made it accessible by the photocleavage of blocking DNA strands.[197] Jäschke and co-workers used nucleobase-caged

residues for the photoactivation of a very different ribozyme, which in this case catalyzes the reaction of two small organic molecules in a Diels–Alder-type reaction.[198]

All these approaches can only be improved by an in-depth understanding of DNA and especially RNA folding. Schwalbe and co-workers spearheaded studies in which laser light sources were coupled to NMR spectrometers for the detailed investigation of the structure and dynamics of nucleic acids. They studied, for example, the Mg^{2+}-dependence of the hammerhead ribozyme's activity and suggested a method to improve the catalytic efficiency.[199] Reversible conformational switching of RNA is especially important for the action of riboswitches—regulatory RNA elements which can control translation and which are perhaps the most beautiful and sophisticated application of RNA folding. Also here, conformational equilibria, for example, of bistable RNAs are important and could be investigated with light-triggered time-resolved NMR spectroscopy by Schwalbe, Pitsch, and co-workers.[200,201] For further details we refer to two excellent review articles.[202,203]

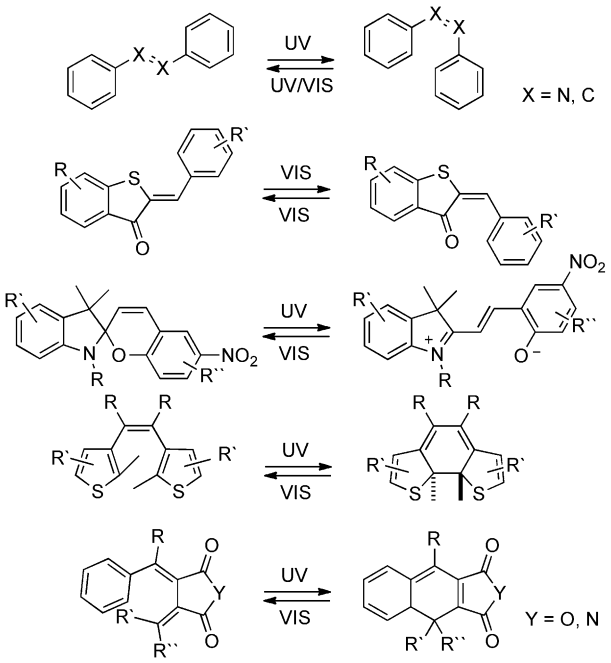
The transition between different functional states is an important field of investigation in DNA-based nanotechnology.[204] Often the developed "walkers" and motors are driven by a manual change in the environmental conditions or by the addition of control oligonucleotides. Since such a fuel source is rather polluting, it is tempting to use light for the generation of the driving non-equilibrium conditions. Although sophisticated approaches have been realized with reversibly photoswitchable oligonucleotides in recent years (see Section 3.4), there are very few studies on caged oligonucleotides. For example, Lukeman and co-workers used an approach in which control oligonucleotides were sequestered on a surface by dense immobilization through a 5′-photocleavable linker and by the formation of an intramolecular hairpin.[205] Photolytically different control strands were liberated from the surface and operated a model machine in a cyclic fashion. In our own contribution we used caged sticky ends to trigger the interaction of DNA minicircles with light.[206]

3. Reversible Photoswitching

Photoswitches have found broad application, especially at the boundary between life and material sciences,[207,208] and a full coverage is beyond the scope of this Review. Topics such as cellular delivery, regulation on surfaces, light-sensitive polymers, and photoresponsive catalysts as well as also the huge body of literature on overcrowded alkenes (which to the best of our knowledge have not been applied in a biological context) will not be considered here.

3.1. Concepts

The light-induced reversible change of color was already observed in the second half of the 19th century.[209] Hirshberg coined the term photochromism for this behavior in 1950.[210] However, it is only in the last 20 years that bistable, photoswitchable compounds have become more and more popular tools for addressing biological questions. There are different classes of organic photochromic systems—mainly undergoing isomerization reactions of double bonds or unimolecular pericyclic reactions (Scheme 2). They can be classified in



Scheme 2. Overview of bistable photoswitches based on azobenzene (X = N), stilbene (X = C), hemithioindigo, spiropyran, diarylethene, fulgide (Y = O), and fulgimide (Y = N; from top to bottom).

terms of the changes that happen upon irradiation. The azobenzene, stilbene, spiropyran, and hemithioindigo (HTI) systems introduce steric changes through switching, and azobenzenes and spiropyrans are especially useful for reversibly creating steric bulk. A change in the conformational flexibility can be generated through switching the diarylethene (DET) and fulgide system. The photochemical properties of these switches in organic solvents have been studied extensively over the last 50 years. However, the aqueous and partly reducing conditions realized in biological systems can dramatically change these properties as well as the stability of photoswitches. Therefore, in the following section an overview is given of these systems and their reported behavior in aqueous systems.

3.1.1. Azobenzene-Derived Photoswitches

The most often used class of photoswitches is by far based on azobenzene, which undergoes a photochemically induced *cis*/*trans* isomerization of the N=N bond. This results in a change from the more stable and planar *trans*-azobenzene to *cis*-azobenzene, in which the two parts of the molecule are tilted by about 55°.[5,211,212] This is accompanied by an increase in the dipole moment from 0.5 D to around 3.1 D and a decrease in the end-to-end distance by about 3.5 Å.[213] The isomerization from *trans* to *cis* is typically accomplished at 350 nm (π-π^* transition), while *cis* to *trans* conversion can be

achieved at 450 nm (n-π* transition) or thermally. As there is a distinct overlap in the UV/Vis spectra of both isomers, complete *trans* into *cis* conversion is not possible photochemically, and irradiation generally produces photostationary states composed typically of 80 % *cis* at best. Conversely, photoisomerization of *cis* to *trans* can only produce up to 95 % of the *trans* isomer.[212,214] In contrast, thermal back-isomerization can yield > 99.99 % of the *trans* isomer.[215] At room temperature, this thermal relaxation occurs within 3 to 4 days for unmodified azobenzenes,[214] but depends strongly on different parameters. As a dipolar character for the transition state of the isomerization has been proposed,[216] increased solvent polarity shortens the half-life time for thermal relaxation.[217–219] A low pH value also accelerates the thermal isomerization, as protonation of the azo bond lowers the activation barrier.[220] Electron-donating and/or electron-withdrawing groups in the *para* positions lead to a stabilization of the transition state and, therefore, to an accelerated relaxation through reducing the N–N double bond character.[221] This was exploited by Woolley and co-workers to maximize the extent of photoswitching by using thermal relaxation for resetting the switch. The azobenzene derivatives **7**[219] and **8**[222] exhibited half-lives of approximately 25 ms and 1 s, respectively (Figure 8). A red-shift of the

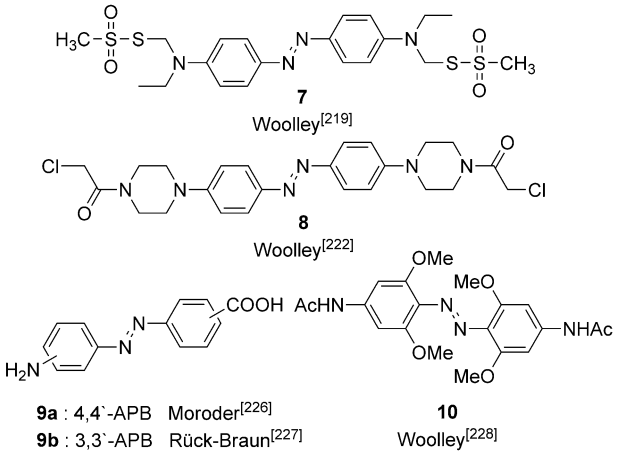


Figure 8. Structures of azobenzene-based photoswitches with partly red-shifted absorption properties for the *trans* isomer and/or increased thermal stabilities of the *cis* isomer.

absorption bands for the *trans* into *cis* conversion with a λ_{max} value of around 480 nm for azobenzene **7** and 450 nm for azobenzene **8** was also achieved, thus permitting the use of less-harmful wavelengths with deeper cell penetration. As a drawback, high irradiation intensity had to be employed—which may damage cells or tissues—to preserve the concentration of *cis*-azobenzene in significant amounts.

Stabilization of the *cis* state can be obtained through substitution at the *ortho* or *meta* position. Asanuma and co-workers reported a half-life for thermal relaxation of > 200 h at 37 °C for a 2,2′-dimethyl-substituted azobenzene (see **23** Figure 14).[223] This stabilization is explained by steric hindrance induced by bulky *ortho* substituents, which results in a distortion of inversion and/or rotation of the azo group during *cis–trans* isomerization. It is also assumed that *ortho* substituents induce a hydrophobic pocket around the azo group which both stabilizes the *cis* structure and destabilizes the polar transition state.[221,224] *Meta* substitution reduces the mesomeric interaction of the substituent with the azo group.[225] Thus, significantly differing half-lives for the azobenzene-based ω-amino acids 4,4′-APB ((aminophenylazo)benzoyl) **9a** ($t_{1/2} \approx 10$ min)[226] and 3,3′-APB **9b** ($t_{1/2} \approx 42$ h)[227] in DMSO were reported. Very recently, the azobenzene derivative **10** with methoxy substituents in all four *ortho* positions was introduced by Woolley and co-workers.[228] This substitution pattern resulted in a thermally stable *cis* isomer with a half-life of 2.4 days in aqueous solution. Another interesting effect was a strong red-shift of the n-π* band of the *trans* isomer, thus resulting in a separation of the n-π* bands of both isomers. This enabled *trans*-to-*cis* photoswitching on application of blue light (530–560 nm) and back-switching with 460 nm, thus avoiding UV light at all. Unfortunately, a tendency towards reduction (glutathione) was observed.

As azobenzenes can generally be prone to reduction by thiols, their stability towards reduction by the tripeptide glutathione (GSH),[229] which is responsible for the intracellular redox potential, has been discussed. Moroder and co-workers analyzed the reduction of an azobenzene-based ω-amino acid by GSH in more detail,[230] and proposed a mechanism in which the thiol group of GSH attacks the azo bond. Interestingly, this reductive attack occurs mainly at the *cis* isomer, in which the azo double bond is less resonance-stabilized through the nonplanar structure. Therefore, electron-donating substituents stabilize azobenzene towards reduction by GSH.[222,224] The presence of GSH was shown to induce an acceleration in the thermal relaxation rate for less-electron-rich azobenzenes.[230,231] In this context, Woolley and co-workers proved the feasibility of azobenzenes for intracellular photocontrol in living organism by using an azobenzene cross-linked fluorescent reporter peptide that was injected into zebrafish embryos. Azobenzene switching could be monitored directly by imaging switching-induced changes of the fluorescence. They could carry out at least 30 photoswitching cycles with no loss of response, and the observed change in the fluorescence remained constant for about two days in vivo. Furthermore, no toxic side effects on the embryo development were evident.[232]

The photochemically induced change in the end-to-end distance is the driving force for functional changes in the target biomolecule, and can be used in particular to control the secondary structure and function of peptides and proteins. Therefore, efforts have been undertaken to increase the end-to-end distance without generating too many degrees of freedom. One strategy to maximize the effect of isomerization was introduced by Moroder and co-workers, who enlarged the switch with acetylene units (compound **11** in Figure 9).[233] Woolley and co-workers designed a water-soluble switch with rigid phenylethynyl units at each side (**12**) which resulted in an average change in the distance of 13 Å.[234] Unfortunately, the photochemical *trans* to *cis* conversion was severely low, probably because of electronic

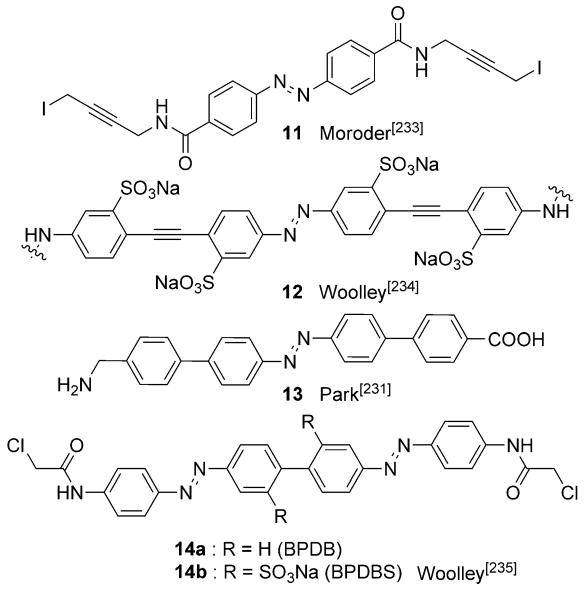


Figure 9. Azobenzene-based photoswitches with increased end-to-end distances.

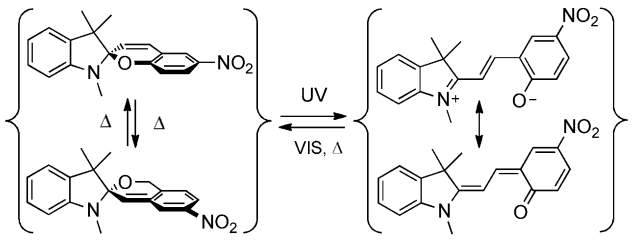
interactions with the phenylethynyl units. Incorporating biphenyl units to increase the end-to-end distance is a strategy that was realized by Standaert and Park.[231] Their derivative **13**, exhibited good photochemical properties and molecular modeling studies revealed a distance change of 13 Å. In a recent study, Samanta and Woolley connected two azobenzene units through a biphenyl linkage, thereby increasing the change in the end-to-end distance to a maximal 23 Å.[235] As irradiation produces *cis*/*trans* isomers as well as *cis*/*cis* isomers, the conformational effects of these switches on target molecules are harder to predict. However, the BPDPS derivative **14b** (Figure 9), with sulfonate groups in the *ortho* position, yielded up to 80% of the *cis*/*cis* isomer in the photostationary state. As Hecht and co-workers have recently demonstrated,[236] this is due to the *ortho* substituents inducing an enlarged dihedral angle between the adjacent azobenzene units. Coupling azobenzene units directly together at the *para* position normally results in an extended electronic conjugation with a dramatically decreased photoreactivity of the switches.[237] The introduction of a large dihedral angle leads to an electronic decoupling of the two azobenzene moieties, thereby improving the photochromic properties effectively (Figure 9). A more comprehensive review about azobenzenes as photoswitches for biomolecules was published recently by Beharry and Woolley,[5] which we want to recommend.

Stilbenes exhibit some advantages over azobenzenes, such as thermal stability of the *cis* isomer and stability towards reductive conditions.[238] However, the need for UV light for the *trans*→*cis* (300 nm) as well as for the *cis*→*trans* (280 nm) conversion has to be considered as the main drawback in the use of stilbenes for biological applications.

3.1.2. *Other Photoswitches*

The class of hemithioindigo (HTI) based photoswitches promises to be an interesting alternative to azobenzenes. Isomerization of the thermodynamically favored *Z* isomer to the *E* isomer by irradiation at 400 nm occurs in yields of around 80%. Both isomers have a planar and unstrained structure.[239] Thus, the *E* isomer exhibits generally higher thermal stability than the *cis* azobenzene. Reisomerization to the *Z* isomer is possible at wavelengths of around 480 nm. The *Z*/*E* absorption bands are more separated compared to azobenzenes, and higher photochemical stability than azobenzenes or fulgides was observed in organic solvents.[240,241] HTI-based ω-amino acids with fast isomerization times (< 10 ps) have been introduced.[242–244] So far, the lability of HTIs towards typical conditions for solid-phase peptide synthesis has limited their application as conformational switches for peptides.[239,245–247]

Spiropyran-type switches, as well as azobenzenes, are mostly used in biological applications. Although the photochemical behavior of spiropyran-type switches is more difficult to predict and more environment-dependent than azobenzenes, the changes in their geometry and polarity through photoswitching are more pronounced. Conjugation to biomolecules is commonly achieved through the N atom of the indole moiety of a spiropyran, but ligation can also be realized at other sites of the spiropyran scaffold, which allows control over the orientation of the switch in the bioconjugate.[248] UV irradiation of a spiropyran leads through ring opening of the bulky colorless spiro (SP) state to the planar and colorful merocyanine (MC) state. The large steric change is accompanied by an increase in polarization from 2–5 D for the SP state to 20 D for the open merocyanine.[249,250] The SP form exhibits a chiral center at the spiro carbon atom, thereby resulting in several possible stable isomers for the open MC state through *cis*/*trans* isomerization about the central double bond. Also, the zwitterionic open form is accompanied by a quionoid uncharged resonance structure, which is presumed to be predominant in hydrophobic environments (Scheme 3).



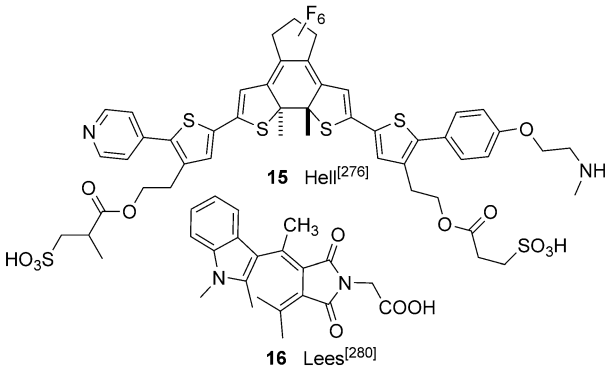
Scheme 3. Isomerization of spiropyran and mesomeric structures of the merocyanine state.

Ring closure proceeds thermally at relatively slow rates, depending on the solvent, or highly accelerated through irradiation with visible light (around 550 nm). The well-separated absorption bands of SP and MC state enables clean switching between the two isomers to be performed. Typically, the thermodynamically stable form is the closed SP state, but

polar substituents such as carboxy, nitro, or sulfonate moieties and a polar environment—especially aqueous solvents—can stabilize the MC state to such an extent that it becomes the thermodynamically favored state in the dark (negative photochromism).[209,251,252] Some other aspects have to be considered when using spiropyrans in aqueous conditions: The quantum yield for the SP-to-MC transition decreases with the polarity of the solvent,[253] but the direct environment in biological systems also strongly influences the switching efficiency. Quantum yields that are comparable to values measured in hydrophobic solvents have been reported for switches immobilized on a protein surface.[254,255] However, Koçer et al. reported a reduced switching efficiency of a spiropyran incorporated in a channel protein.[256] Tethering nitrospirobenzopyran to an oligonucleotide—either through a short linker to the 5′ end[257] or internally to a 2′-OH[258] group (see **28** Figure 14)—resulted in a loss of photoactivity for transforming the SP state into the MC state. In contrast, Andréasson and co-workers observed not only photoreactivity but also intercalation of the MC form into a DNA double helix after UV irradiation, when incubating calf-thymus DNA with nitrospirobenzopyran.[259] Thus, the photochemical behavior is sometimes hard to predict for spiropyrans. The hydrolytic stability of spiropyrans is also considered an obstacle for biological applications, as rapid decomposition of spiropyrans in aqueous buffer at 60 °C[260] and in basic conditions[257,258] was observed. This hydrolytic degradation occurs mainly at the open merocyanine state through a retro-aldol reaction. However, other studies suggest the rate is again environment-dependent, with the reversible switching of spiropyrans repeated more than 10 times without significant degradation both in vitro[254] and in vivo.[261] A recent study by Giordani and co-workers[262] concerning the toxicology of spiropyrans showed that micromolar concentrations of spiropyrans induce only slight cytotoxic effects in various cell lines. The hydrophobic SP form, in contrast to the polar MC form, was also observed to be cell-membrane permeable.[263,264] Marriott and co-workers reported the possibility to efficiently switch the SP state of nitrospirobenzopyrans to the MC state in aqueous conditions upon two-photon excitation at 720 nm.[265,266] This is in contrast to azobenzenes, which have only small two-photon cross-sections, thus offering the opportunity to use spiropyran-based switches within tissue samples and animals.

Diarylethenes and fulgides/fulgimides are hexatriene derivatives that undergo photoinduced reversible electrocyclic ring closure and ring opening.[267,268] Both classes feature negligible thermal relaxation and diarylethenes, in particular, exhibit high fatigue resistance. Only recently, diarylethenes and fulgimides have become more and more interesting for biological applications—particularly diarylethenes for high-resolution microscopy. The two isomers of diarylethenes possess very different absorption spectra that allow switching rates of generally over 90% conversion from the colorless and flexible open form to the delocalized and, therefore, deeply colored and rigid closed form.[269] The cyclization and/or cycloreversion of some diarylethenes can also be induced electrochemically,[270] and the synthetically straightforward spectral tunability with different substituents allows the wavelength for cycloreversion to be shifted from around 500 nm to over 700 nm.[271]

Recent studies show that the excellent fatigue resistance and high thermal stability of diarylethenes are also maintained in aqueous environments.[272,273] However, the low water solubility can be a limiting factor. Therefore, diarylethenes have been decorated with amphiphilic side chains,[274,275] but this results in self-association of these molecules in aqueous solutions. Recently, Hell and co-workers introduced water-soluble diarylethene **15**, which shows a good switching performance in water and has the possibility to conjugate to other molecules of interest through a secondary amino function (Figure 10).[276]



15 Hell[276]

16 Lees[280]

Figure 10. Water-soluble diarylethene- and fulgimide-based photoswitches.

The colorless isomer of a fulgide in the *E* conformation undergoes cyclization upon UV irradiation to give the colored closed form, which possesses an extended π system and is almost planar.[268,277] *E*-*Z* isomerization of the double bond can also occur during UV irradiation. A drawback of fulgides is that ring closure can only proceed from the *E* isomer. Complete reopening can be achieved through irradiation with visible light around 500 nm and proceeds, similar to the ring closure, on a ps time scale, although quantum yields are decreased in polar solvents.[278–280] Fulgides hydrolyze in aqueous environments because of their anhydride structure; therefore, hydrolysis-resistant fulgimides are used for biological applications. Their succinimide moiety also allows facile functionalization of the photoswitch. However, only a few studies concerning the properties of fulgimides in aqueous environment have been reported. In an early study, Willner et al. attached a fulgimide derivative to concanavalin A, which was stable in aqueous solution for 48 h at 25 °C and could be cycled back and forth twice.[281] In 2004, Rentzepis and co-workers demonstrated that an indolylfulgimide derivative that also exhibited fluorescence in its open state was able to enter cells by diffusion.[278] Switching back and forth could be repeated at least seven times in vivo. Only recently, more studies have been undertaken by the research group of Lees to optimize fulgimides for aqueous systems.[280,282] For example, fulgimide **16** exhibited excellent thermal stability in buffer with approximately 3% degradation for the open and about 20% degradation for the closed form after 500 h at

37 °C. Photochemical-induced cyclization of compound **16** produced up to 87 % of the closed isomer, and switching could be performed around 80 times before degrading by 20 %. Although not yet widely used, these examples show that fulgimides bear the potential for applications in a biological context.

3.2. Photoswitchable Small Molecules

Molecules for the reversible regulation of enzyme activity by light have been developed, as they promise a sophisticated level of selective control in organisms. Kuhn and co-workers realized the similarity of a hemithioindigo to some inhibitors of 12/15-lipoxygenases (LOXs). The inhibitory potency of the *E* isomer for rabbit 12/15-LOX proved to be more than 33-fold higher than that for the *Z* isomer, but the high IC_{50} value of the *E* derivative (0.021 mM) limited its use as a LOX inhibitor.[283] Δlac-acetogenin, an inhibitor against complex I in the respiratory chain of mitochondria, was modified with azobenzenes (**17**, Figure 11).[284] Reversible changes in enzyme activity were observed, but complete recovery of activity was not possible because of incomplete *cis* to *trans* photoisomerization. Reversible photoswitching of a surface-attached azobenzene decorated with a phenylalanine-based trifluoromethylketone inhibitor resulted in light-regulated binding of α-chymotrypsin to a surface.[285] Fischer and co-workers showed that the influence of irradiation on the inhibitory efficacy of the azobenzene-modified immunosuppressant cyclosporine A could be optimized by augmenting the structural differences between the photoswitchable conformers by applying the "protein-borrowing" strategy.[286,287] The human carboanhydrase I (hCAI) inhibitor benzenesulfonamide and a copper(II)–iminodiacetate (IDA) complex, which binds to histidine residues close to the active side of the enzyme, were connected over a DET switch (**18**).[273] Switching from the closed to the open form with visible light reproducibly reduced the IC_{50} value by about 55-fold to 8 nM in vitro. In another study by Branda and co-workers, DET derivative **19** was taken up orally by *C. elegans* and retained its photoactivity inside the nematode for at least three cycles. As a consequence of the lower reduction potential of the closed form and its probable interruption of the metabolic electronic pathways, the closed form induced paralysis of the nematodes, while switching to the open form restored the mobility of the organisms.[288]

The regulation of cell signaling has also been addressed with azobenzenes by Trauner and co-workers.[289,290] They designed a whole class of nontethered photochromic ligands (PCLs) to regulate the action potentials of ionotropic glutamate receptors and voltage-gated K^+ channels. Light-dependent neuronal firing of iGluR5 and iGluR6 receptors in HEK 293 cells was achieved by using an azobenzene-substituted glutamate agonist.[291] The class of membrane-permeable azobenzenes connected to a quaternary ammonium group, which mimics the derivative QX-314 from the analgesic lidocaine, functions as photochromic open channel blockers by binding to the internal tetraethylammonium (TEA) binding site of the channel (see Figure 11). They can block either in the *trans* (**AAQ**,[292] **BzAQ**,[293] **DENAQ**[294]) or in the *cis* state (**PrAQ**,[293] **PhENAQ**[294]). Photochemical control of K^+ channels in HEK 293 cells, Purkinje neurons, and heart central pattern generator interneurons (HN cells), which regulate the frequency of heart contractions, was demonstrated.[293]

The function of oligonucleotides depends strongly on their secondary structure. Therefore, photoswitches have been utilized for DNA and RNA binding as well as structure modulation. Baigl and co-workers designed a class of azobenzene-based cationic surfactants as sequence-independent condensing agents for DNA[295,296] which induced DNA compaction in the *trans* state and DNA unfolding in the *cis* state. Regulation of the transcription and translation of GFP with these molecules was shown in vitro.[297] Mismatch binding ligands consisting of two base-recognition elements based on naphthyridines and optionally azaquinolones connected

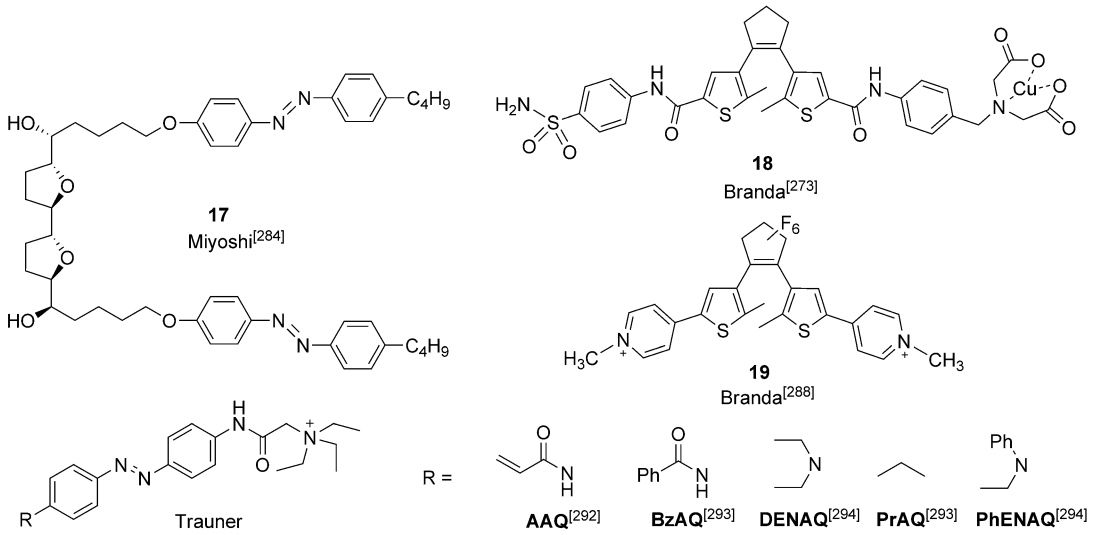


Figure 11. Photoswitchable inhibitors and regulators.

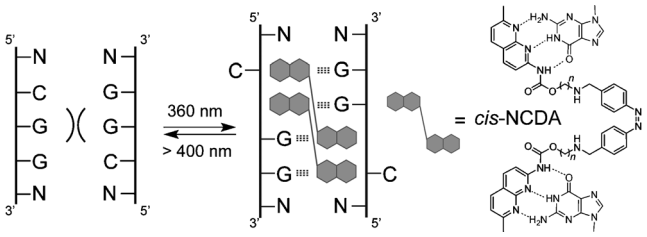


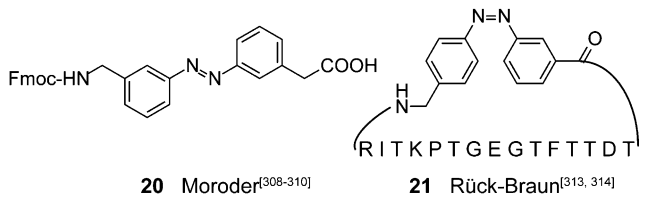
Figure 12. Photoswitching of DNA hybridization with azobenzene-based molecular glue NCDA ($n = 2,3,4$).[298–300]

through an azobenzene were used as a reversible molecular glue for DNA hybridization by Nakatani and co-workers (Figure 12). In the *cis* form, hybridization was caused by ligand binding to GG or GA mismatches. Switching to *trans* azobenzene led to a change in the orientation of the base-pairing moieties, thereby resulting in dehybridization.[298–300] With the long-term objective to design reversible, light-switchable gene elements, the selection of short oligonucleotides—so-called aptamers[169]—against one isomer of a photoswitch has been attempted by using the SELEX[169] approach. Aptamers against nitrospirobenzopyran,[301] an arginine-substituted azobenzene derivative,[302,303] and dihydropyrene[304] have been described, but the K_D values obtained still lie within the lower μM range. In the last case, the selected aptamer was also connected to a hammerhead motif to create a reversible light-regulated ribozyme.

3.3. Photoswitchable Peptides and Proteins

Photoswitches, mostly azobenzenes, have been used extensively to analyze the folding and unfolding of peptide conformations by CD-, NMR-, and time-resolved optical spectroscopy as well as MD simulations.[5,305–307] Since 2006, new studies have been published on the control of β-hairpin structures. Moroder and co-workers analyzed the β-hairpin folding of the tryptophan zipper motif with azobenzene **20** acting as a β I′-turn mimetic in the *cis* form (Figure 13).[308–310] Hilvert and co-workers also used this azobenzene linker to efficiently regulate the hairpin folding of the 36 mer avian pancreatic polypeptide.[311] Photocontrolled triple-helix folding and unfolding was archived by Moroder and co-workers by cross-linking thiol functions of a collagen peptide with the rigid azobenzene clamp **11**.[233,312]

Rück-Braun, Beyermann, and co-workers developed an azobenzene-containing cyclic peptide that mimics the β-finger motif in neural NO synthase (see Figure 13). This motif is crucial for the interaction with the PDZ domain of α-1-syntrophin. While *trans*-**21** showed no affinity for the PDZ domain, isomerization to the *cis* isomer yielded a remarkable affinity of the peptide for syntrophin ($K_D = 10.6$ μM).[313] Thus, *cis*-**21** led to an inhibition of the NO synthase/synthropin interaction, thereby resulting in light-controlled muscle fiber contraction because of a reduced release of NO from skeletal muscle cells; this effect was also nicely demonstrated in vivo.[314]



20 Moroder[308-310] **21** Rück-Braun[313, 314]

Figure 13. An azobenzene-based amino acid used for the analysis of β-hairpin folding and a PDZ domain mimicking cyclic peptide for photoregulation of the NO synthase/syntrophin interaction.

In 2009, Woolley and co-workers demonstrated that screening a protein crystal structure for suitable mutagenesis and cross-linking sites at the FynSH3 domain can be an effective method for the modulation of larger peptides or even globular proteins with an azobenzene photoswitch.[315] Engineering cross-linking sites into voltage-gated ion channels or ionotropic glutamate receptors was also widely used by the research group of Trauner to very impressively photoregulate receptor functions by agonists that were covalently tethered through an azobenzene moiety. For this topic we refer to a review recently published by this research group.[316] Pingoud and co-workers generated photoswitchable variants of the restriction enzymes scPvuII[317] and SsoII.[318] An up to 16-fold increase in DNA cleavage activity could be achieved for PvuII upon irradiation by introducing maleimidoazobenzene derivatives close to the active site. Driessen, Feringa, and co-workers showed reversible photocontrol of protein translocation by cross-linking the lateral gate of channel protein SecYEG with an azobenzene linker.[319]

3.4. Photoswitchable Oligonucleotides and Nucleic Acids

In most cases azobenzenes have been applied for the reversible photoregulation of oligonucleotides. The system of Asanuma, Komiyama, and co-workers, with D-threoninol used as a scaffold to tether azobenzene to DNA[320] and RNA[321] (**22**, Figure 14), has found broad application, for example, for the photoregulation of transcription[322,323] or DNA ligation.[324] Its building block for the solid-phase synthesis of oligonucleotides is already commercially available. This approach is based on the additional insertion of residue **22** between the bases, with all the other residues maintained. While *trans*-azobenzene stabilizes a DNA duplex through stacking interactions,[325] isomerization to the *cis* state results in duplex destabilization, as indicated by a decreased melting temperature of the duplex. Multiple residues have to be inserted (i.e. nine residues for a 20 mer oligonucleotide) for an efficient photoregulated dissociation of a duplex.[326] Asanuma et al. also emphasized the importance of temperature for UV isomerization, because of the moderate photoisomerization rates of the inserted switches. Operation temperatures between the melting temperatures of the duplex in the *trans* and in the *cis* forms optimize the efficiency.[327] Another strategy to improve the photoisomerization efficiency was demonstrated by Tan and co-workers. Adding silver nanoparticles enhanced the closed to open conversion of their DNA nanomotor[328] containing residue **22**

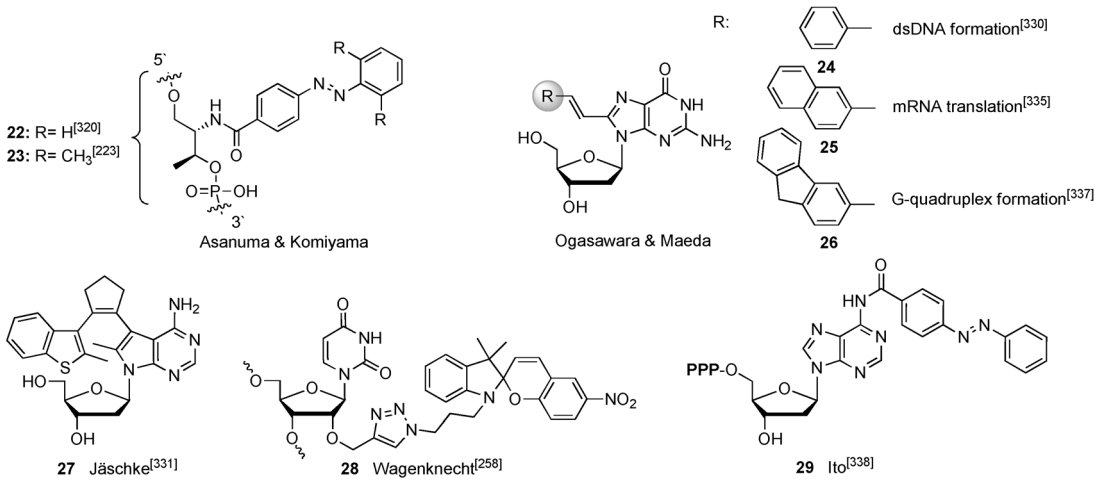


Figure 14. Reversibly photoswitchable nucleotide replacement systems for the photoregulation of oligonucleotide hybridization.

from around 20% to up to 85%.[329] By using a 2′,6′-dimethylazobenzene residue (**23**) instead of the unmodified azobenzene, Asanuma and co-workers could increase the half-life time of the *cis* form by about 10-fold (see Section 3.1) and also the ΔT_m value threefold.[223]

Attempts to use other photoswitchable systems for the reversible regulation of duplex hybridization have also been undertaken. Ogasawara and Maeda introduced photoswitchable deoxyguanosine derivatives modified at C8 with arylvinyl substituents (**24**–**26**, Figure 14). This approach does not require additional residues to be inserted into the oligonucleotide. Enlargement of the aromatic substituent resulted in favored, red-shifted wavelengths for the *trans*/*cis* isomerization. However, the styryl-substituted derivative **24** already slightly destabilized a duplex in the *trans* state.[330] Singer and Jäscke synthesized the diarylethene-functionalized 7-deazaadenosine derivative **27**, but have not yet integrated this system in DNA.[331] Beyer and Wagenknecht introduced a spiropyran into DNA by a click reaction (**28**, Figure 14), but unfortunately it was no longer photoactive.[258]

Stafforst and Hilvert introduced azobenzene-modified peptide nucleic acids (PNAs) for the manipulation of PNA/DNA hybridization. They nicely demonstrated photocontrolled inhibition of DNA transcription by using triplex-forming PNAs.[332] Two interesting new approaches to reversibly photoregulate RNA cleavage have been published. Komiyama, Kuzuya, and Tanaka demonstrated the regulation of acridine-assisted RNA scission through Lu^{3+} ions by incorporating an acridine and an adjacent azobenzene residue in a DNA counterstrand.[333] Site-selective RNA activation by acridine was reduced through stacking interactions when the azobenzene residue was in the *trans* state, whereas switching to the *cis* isomer stopped the acridine from stacking. They estimated a 14.5 times higher intrinsic activity for the *cis* isomer. Complete on-off photoswitching of RNA cleavage was achieved with a different design by Asanuma and co-workers.[334] They attached complementary azobenzene-containing sequences to both ends of a DNAzyme. After hybridization, these strands formed an interstrand-wedged duplex, thereby masking the cleavage site of the DNAzyme. Dissociation of the duplex was induced by a *trans* to *cis* isomerization of the azobenzene residues and restored the DNAzyme activity.

Ogasawara and Maeda could efficiently control mRNA translation of GFP by using a photoresponsive 8-naphthylvinyl-guanosine 5′ cap.[335] In the *trans* form, this cap completely inhibited translation because of steric hindrance between the substituted residue and the active center of eukaryotic initiation factor 4E. Producing the *cis* form with light of 410 nm resulted in GFP translation with the same efficiency as achieved with the normal 5′ cap.

Photoresponsive oligonucleotides have also been used to control G-quadruplex structures and hence applied to the regulation of a thrombin-binding DNA aptamer (see also Section 2.4.2). This aptamer forms a G-quadruplex in the active state and inhibits thrombin-mediated coagulation through binding to exosite 1. Tan and co-workers attached azobenzene-modified complementary DNA to the aptamer sequence through a polyethylene glycol linker.[336] When the azobenzene residues were in the *trans* state the complementary domain hybridized to the aptamer sequence, thereby resulting in a disruption of the G-quadruplex structure and aptamer function. Switching to *cis*-azobenzene restored the aptamer structure and prevented blood clotting. By substituting a dG in the quadruplex structure by 8-fluorenylvinyl-2′-deoxyguanosine **26**, Ogasawara and Maeda could also demonstrate reversible photoregulation of the aptamer–thrombin interaction.[337]

In an interesting approach, Ito and co-workers applied the photoresponsive adenosine triphosphate **29** instead of natural ATP to an in vitro selection of an RNA aptamer for hemin.[338] They could isolate a hemin-binding aptamer sequence, thus demonstrating that compound **29** is a suitable substrate for T7 RNA polymerase, and the resulting RNA serves as a template for reverse transcription. However, only modest photoresponsive binding behavior was observed, probably because of incomplete *trans* to *cis* isomerization.

DNA as a self-assembling material has also become more and more interesting for the development of smart nanostructures and nanodevices. An external trigger, such as DNA

control strands or ATP hydrolysis, is generally required to induce structural changes or three-dimensional movements in these structures, but this has the shortcoming of producing waste. To overcome this, and because of their precise temporal and spatial controllability, photoresponsive oligonucleotides have also been applied for this purpose. Tanaka et al. synthesized light-controllable DNA capsules by inserting azobenzenes into the sticky ends of three-point-star motifs. These capsules were stabilized through stacking interactions of the azobenzenes; however, breakdown of the structure could be readily achieved through photoisomerization.[339] Tan and co-workers reversibly controlled the extension and contraction of a tetrahedral DNA structure by photoregulated binding of an azobenzene-modified single strand to an internal hairpin structure in the tetrahedron.[340] It was also demonstrated by this research group that the photoregulated hybridization of complementary azobenzene-modified DNA oligonucleotides can be used to modulate the orientation and proximity of enzymatic assemblies. This was shown exemplarily for the glucose oxidase/horseradish peroxidase (HRP) multienzyme system and for the concatenation of glucose oxidase with the HRP-DNAzyme.[341]

A further step towards the development of more sophisticated photoswitchable nanodevices was very recently achieved by Asanuma and co-workers.[342] They combined two differently substituted azobenzene residues in a DNA nanomachine to produce repetitive seesaw-like motions upon irradiation with light of different wavelengths (Figure 15). This is, to the best of our knowledge, also the first example of the wavelength-selective activation of different azobenzene switches in one sample.

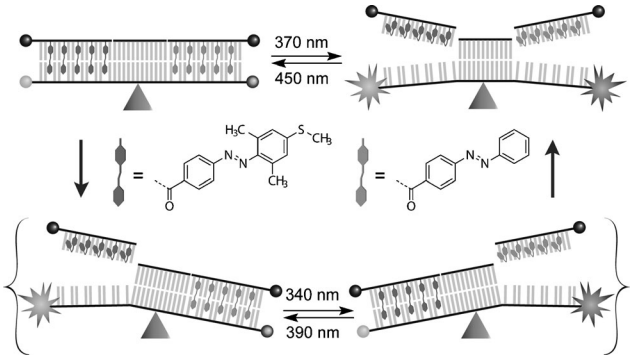


Figure 15. Seesaw-like motion of a photon-fueled DNA nanodevice could be achieved by incorporating two different azobenzene moieties. Whereas *trans*-2,6-dimethyl-4-(methylthio)azobenzene-4′-carboxylic acid photoisomerizes with visible light, unsubstituted *trans*-azobenzene-4′-carboxylic acid requires irradiation with UV light. Complete dissociation and rehybridization was realized with light of 370 nm and 450 nm.[342]

4. Optogenetics

Optogenetics is the collective term that describes techniques developed in recent years that aim to control cellular activity, particularly in neurons, by using light ("opto") and genetically encoded photosensitive actuator proteins ("genetics", Figure 16). This contrasts with the approaches discussed in Sections 2 and 3, where chemical synthesis was necessary. However, this delineation has been softened by approaches called optochemical genetics, which are basically a combination of both worlds. Further discussion on this theme can be found in very recent review articles.[316,343] As a consequence of its totally different approach, optogenetics offers significantly different scopes. For example, in live animals, optogenetics permit assessing the roles of single neurons or neuronal ensembles in the control of a behavior or aspects thereof. The ability to genetically encode the light-sensitive proteins furthermore allows only the cells of interest, even single neurons, embedded within a dense network of other cells or neurons to be specifically addressed. Even if genetic techniques do not allow the optogenetic protein to be expressed in only the cell of interest, technical advances allow illumination of this cell or cell type to be restricted or to be delivered deep within tissue by microfabricated light fibers. It should be mentioned that the imaging of cellular activity by using genetically encoded photosensors is also often summarized under the concept of optogenetics; however, imaging will not be part of this Review and the reader is referred to numerous excellent reviews on this topic.[344–346]

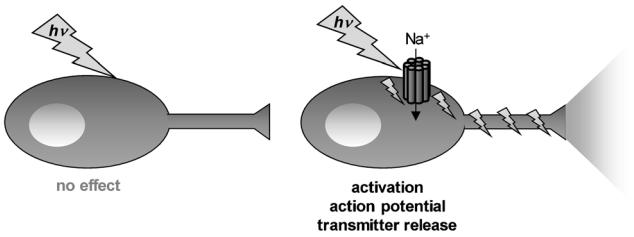


Figure 16. Principle of optogenetics: Heterologous cells, particularly neurons, which normally do not respond to light (left), are rendered light-sensitive ("opto") by targeted expression of a photosensitive protein of heterologous origin ("genetics"; right). In this case, a light-activated cation channel mediates depolarization of the neuron, thereby causing action potential firing and, consequently, transmitter release.

The basic idea of optogenetics was adopted by several researchers, and several approaches to the problem were taken.[343,347,348] The most prominent and, because of their simplicity, most applied tools for the control of membrane potentials are natural photosensor proteins of the rhodopsin family. Channelrhodopsin-2 (ChR2), a blue-light-gated cation channel,[349] and halorhodopsin, a yellow-light-driven Cl^- pump,[350] are used, in particular, to achieve photo-depolarization and photo-hyperpolarization, respectively (Figure 17). Since the first demonstrations that channelrhodopsins are directly light-gated ion channels in 2002 and 2003,[349,351] initial proof-of-principle technical reports appeared in 2005 that demonstrated the utility of ChR2 to depolarize neurons in culture,[352] as well as in live animals,[353,354] where this could even elicit specific behavior.[355] These tools are now being widely applied, particularly in the neurosciences, and have been further modified (see below). A number of studies have introduced additional optogenetic tools to affect membrane potential, protein–protein interactions, and intracellular signaling.[356–363] As mentioned before, a complementary

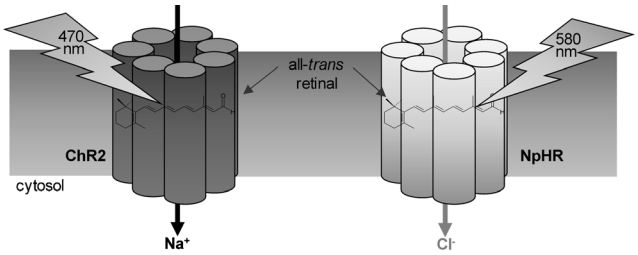


Figure 17. Channelrhodopsin (ChR2) and halorhodopsin (NpHR) are microbial rhodopsins that are widely used to achieve exogenous, multimodal control of neuronal activity. Channelrhodopsin, a cation channel that transduces mostly Na^+ under physiological conditions, can be used to induce photo-depolarization, by using blue light (left), while halorhodopsin, a yellow-light-driven, inward-directed Cl^- pump mediates photo-hyperpolarization. Both proteins require all-*trans* retinal as the chromophore.

approach in "optochemical genetics" is to use chemical biology to attach light-switchable ligands to genetically modified ion channels so as to achieve optical control—these have been elegantly reviewed in recent publications[316,343] and will not be covered here. In essence, optogenetics aims at no lesser a goal than to understand the role of neurons within circuits by influencing behavior or higher brain functions, by stimulating or inhibiting defined neurons or neuronal populations with light, and is essentially non-invasive and on a neuronal timescale. In addition, the imaging of neuronal activity using genetically encoded sensors allows the activity of many neurons to be monitored in parallel and can, to some extent, be combined with optical methods to stimulate or inhibit cellular activity. Thus, in the past few years, optogenetics has revolutionized research in the neurosciences (i.e. on synaptic transmission, neural circuits, whole nervous systems, and behavior) and in more-general cell biology. Publications involving the use of optogenetic techniques are skyrocketing (>300 at the time of writing this review), we thus cannot claim to comprehensively describe the field and apologize to any colleagues whose work we may not have been able to mention here.

4.1. Methods To Stimulate or Inhibit Neurons by Altering the Membrane Potential Using Genetically Encoded Sensors of Heterologous Origin

4.1.1. Drosophila Photoreceptor Cascade and Uncaging of Ligands for Orthogonal Receptors

In 2002, Miesenböck and co-workers presented a study that may be considered the birth of optogenetics as we understand it today.[364] They "transplanted" the photoreceptor cascade of the *Drosophila* eye into vertebrate neurons. Their first trials involved transferring 10 different genes, but later they could reduce the requirement to co-expressing arrestin, rhodopsin, and the cognate α subunit of the heterotrimeric Gq protein ("chARGe") to stimulate the cells. In vertebrate neurons, this system responds to white light, activating the rhodopsin, which would then recruit the *Drosophila* Gαq protein together with the vertebrate G β and γ subunits. The activated G proteins apparently coupled to phospholipase C, thereby generating inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) as secondary messengers, which induced membrane currents (depolarizing the cell) and elevated intracellular Ca^{2+} levels, thus triggering synaptic transmitter release. This was a seminal study, which demonstrated the concept of optogenetics for the first time. However, this system was not widely applied, as it was comparatively slow, inducing neuronal spiking only after several seconds. Furthermore, neuronal activity stopped only slowly in the dark. To overcome these shortcomings, but still use light as a fast trigger for neurons and also to maintain the genetic encodability, Miesenböck and co-workers expressed ligand-gated ion channels foreign to the host organism (vertebrate neurons and fruit flies). They used purinergic P_2X_2 receptors or transient receptor potential vanilloid (TRPV) channels, and then photo-uncaged the ligands of these channels (ATP or capsaicin, respectively). In this way, rapid spiking and even behavioral changes could be invoked.[362,363] However, the need to introduce caged ligands into live animals somewhat limits the applications of these tools.

4.1.2. Microbial Rhodopsins

In 2002 and in 2003, Nagel, Hegemann, Bamberg et al. demonstrated that microbial rhodopsins from *Chlamydomonas reinhardtii*, well known for their involvement in the phototaxis of these green algae, were acting directly as light-gated cation channels.[349,351] Thus, the two proteins were renamed channelrhodopsins (ChRs); ChR1 was shown to be more permeable to protons, and ChR2 also showed a clear conductivity for Na^+ and to some extent Ca^{2+}, both being maximally activated by blue light (ChR1 ≈ 500 nm; ChR2 ≈ 470 nm). While these findings first met a lot of skepticism, it was soon recognized how remarkable they actually were, as they opened up the whole field of optogenetics in a widely applicable and experimentally very straightforward way. Particularly ChR2, which functioned as a light-gated channel even when truncated after amino acid 315, expressed well in heterologous cells. Nagel et al. could show that "foreign" systems, such as *Xenopus* oocytes and HEK293 cells, could be photo-depolarized when expressing ChR2 (Figure 17).[349] Furthermore, ChRs require only all-*trans*-retinal as a cofactor, which later proved to be present in mammalian tissue in sufficient amounts, or could be easily added to the culture media for cells or even whole animals (nematodes, fly larvae). So in 2003 Nagel et al. proposed that "… *expression of ChR2 in oocytes or mammalian cells may be used as a powerful tool to increase cytoplasmic Ca^{2+} concentration or to depolarize the cell membrane, simply by illumination*."[349]

Channelrhodopsin-2 was recognized by several researchers in the neurosciences as a potentially highly useful tool to photostimulate neurons, and the first successful examples of applications in neurons were reported in 2005: Boyden and Deisseroth published a study in the summer of 2005[352] in which they reported the photo-depolarization of rodent neurons in culture and in brain slices, which caused the cells

to spike. In December 2005, Hegemann, Landmesser, Herlitze, and co-workers showed the same effect, including evoking muscular twitching responses, in hippocampal neurons as well as chick spinal cords and even embryos,[353] while Nagel, Bamberg, Gottschalk et al. demonstrated the photocontrol of muscles, neurons, and behavior in the nematode *C. elegans*, mediated by ChR2, also in December 2005.[355] In 2006, Yawo and co-workers showed that ChR2 photoactivation can depolarize neurons in live mice,[354] while Pan and coworkers demonstrated the expression of ChR2 in inner retinal neurons (bipolar cells and retinal ganglion cells) in a mouse model of *Retinitis pigmentosa*.[365] These animals were blind due to photoreceptor degeneration; however, in these animals, signals generated by the application of light to the retina were indeed transmitted to the visual cortex, thus indicating that some optical signaling could be restored. Also in 2006, a first application in *Drosophila* larvae showed the feasibility of ChR2 to induce certain behaviors,[366] and in 2008, a publication by Engert and co-workers demonstrated ChR2-induced behavioral changes in zebrafish.[367]

More prominent applications of ChR2 in the rodent brain followed. In 2007, a transgenic mouse expressing ChR2 in different neuronal populations, and the first stimulation of (olfactory) neurons in a live mouse was reported by Feng and co-workers.[368] The Svoboda research group used ChR2 to stimulate neurons in mouse brain slices to functionally map long-range connections,[369] and Oertner demonstrated optical induction of synaptic plasticity.[370] In addition, the stimulation of neurons in the primary motor cortex was reported, where thin fibers were utilized to guide light into the skull of an animal—with spectacular effects: By some (poorly understood) mechanism, photoactivation of cells in the right hemisphere of the motor cortex immediately caused mice to circle clockwise, while illuminating the left hemisphere resulted in counterclockwise rotations—until the light stimulus was ended.[371]

In 2007, another milestone in optogenetics was reported: Two studies, one by the Boyden research group,[372] the other as a result of a joint effort by the Bamberg, Gottschalk, and Deisseroth groups,[350] demonstrated the use of halorhodopsin (NpHR) from *Natronomonas pharaonis*, a yellow light-driven Cl^- pump, to hyperpolarize neurons in culture and in brain slices, as well as in live *C. elegans* and thus instantaneously block neuron-provoked behaviors (Figure 17). Furthermore, they showed that NpHR and ChR2 could be simultaneously, but independently, used to hyper- or depolarize cells, thus achieving bidirectional control. Halorhodopsin has since been applied in several elegant studies, among them the generation of a transgenic mouse that permanently expresses a trafficking-enhanced version of halorhodopsin,[373] and also in the retinae of *Retinitis pigmentosa* mice, where it could be used to hyperpolarize the remaining photoreceptors, thus mimicking the light response.[374] As an addition to the set of hyperpolarizers, the Boyden research group established the use of microbial outward-directed proton pumps, particularly archaerhodopsin ("Arch") from *Halorubrum sodomense* and the proton pump from the fungus *Leptosphaeria maculans* ("Mac").[375] Lastly, optogenetic tools have also been generated for expression in primates, which function to activate or inhibit neuronal spiking, although, however, no macroscopic behavior could thus far be provoked in primates.[376,377]

Many researchers have been attracted by the utility of these optogenetic tools, and started applying them for various purposes, not only for cellular activation, but also to study synaptic plasticity[370] or chemical transmission at synapses.[378,379] Through the use of microbial rhodopsins, it was quickly recognized that these tools were not perfect, and that additional tools would be useful. Thus, several researchers have explored the diversity of nature to identify further rhodopsins, for example, with different spectral response properties, and achieve red-shifted excitation.[380,381] In addition, ChR2 has been modified or mutated to yield proteins with different kinetics,[382–385] ion conductance, for example, of Ca^{2+},[386] or enhanced currents and/or expression.[387] Recently, the functional fusion of two rhodopsins was demonstrated, which allows highly controllable optical excitation and inhibition to be achieved in the same cell, as the relative expression levels of the de- and hyperpolarizing actuators are fixed.[388] The recent crystal structure of a chimera of ChR1 and ChR2 is likely to guide the development of additional ChR variants with different ion conductances or specificities as well as spectroscopic or kinetic properties.[389]

Many more technically driven studies were aimed at different aspects of the practical use of rhodopsin-based optogenetics: The application of light to neurons deep within the rodent brain was realized by implanting thin light fibers[371,390] or more complex devices that allow the light to be transported to several sites and depths within the brain.[391] Excitation with red or infrared light would be desirable to penetrate deeper into living tissue, and thus several studies concerned the two-photon excitation of ChR2.[392,393] Other researchers worked out ways to illuminate distinct neurons in freely moving transparent nematodes, to invoke (single) cell-specific behavioral effects.[394,395] Complex patterns of light were also used in mammalian neurons to stimulate only parts of a cell, or to recruit as many receptors as possible in the small area of the cell body of a neuron.[392,393,396]

Lastly, the utility of rhodopsins as therapeutic tools or tools for medical research are being explored. The first attempts to realize a visual prosthesis in a model of *Retinitis pigmentosa*, based on channelrhodopsin or halorhodopsin have been presented.[365,374,397] Optogenetic tools helped to identify neurons that can be stimulated to alleviate Parkinson's symptoms in a rodent model,[398] to re-instate breathing in spinal cord injury,[399] and the optical pacing of cardiac muscle tissue has also been demonstrated.[400]

4.2. Methods To Affect Intracellular Signaling/Secondary Messenger Pathways

Initial success in optogenetics based on rhodopsins and other light-sensitive proteins of heterologous origin sparked an interest in harvesting additional proteins from nature to affect cellular processes in heterologous cells. Secondary messenger pathways are crucial for countless processes in cellular biology, ranging from determining membrane poten-

tials, ionic concentrations, cell motility to gene expression, and cell growth or division, often in response to exogenous signaling molecules. Thus, such pathways were targeted using optogenetics. For example, G-protein-coupled receptors (GPCRs) generally activate intracellular heterotrimeric G proteins, which activate effector proteins to generate small-molecule secondary messengers. As the natural photosensor of the retina, rhodopsin acts as a GPCR. It was used to drive secondary messenger pathways in cells other than photoreceptors which respond to activation of the same Gα ($G_{i/o}$) protein that rhodopsin couples to.[353] This concept was later expanded by replacing the cytosolic domains of rhodopsin with the respective domains of other GPCRs that couple to different G proteins, thereby generating so-called "opto-XRs".[358]

Another optogenetic approach uses light-activated enzymes to directly produce a secondary messenger, namely cyclic adenosine monophosphate (cAMP). This photoactivated adenylyl cyclase (PAC) was used to trigger neuronal responses in fruit flies and to enhance synaptic transmission in *C. elegans*. In contrast to the strong photostimulation by ChR2, it does not override intrinsic programs of the nervous system, and thus provides an additional quality in optogenetic control.[356,357] A more potent photostimulated adenylyl cyclase has since been identified and used in mammalian neurons.[401] A similar enzyme for the production of cGMP has been generated by mutagenesis, but not as yet tested in animal cells.[402]

Intracellular signaling in response to extracellular ligands often involves triggered protein–protein interactions. Such interactions have been rendered photosensitive, by masking protein interaction domains with the LOV (light, oxygen, voltage) photoswitchable protein domain. This small protein domain contains a blue-light-sensing flavin chromophore, which upon illumination induces a conformational change that frees an α helix from the flavin binding domain. This unfolding can be used to unmask a protein–protein interaction domain on a protein to which the LOV domain is fused through the α helix. In this way, the small G proteins rac and cdc42, both involved in modulating cellular motility and cytoskeletal remodeling, have been addressed, thus allowing control over cellular shape and motion.[359] This approach was also used successfully to generate a light-triggered, one-component, gene-expression system.[403] Other photosensitive, genetically encoded systems that allow protein–protein or protein–ligand interactions to be controlled in vivo have also been developed.[360,361]

5. Outlook

As we have demonstrated, there are three very distinct types of strategies available for the regulation of (bio)molecular processes with light, each with their own specific advantages and disadvantages. Uncaging is at least conceptually easy to realize but inherently irreversible. However, if the functional groups of a molecule which are responsible for its activity are properly caged and if the uncaging process is clean, excellent on/off ratios can be obtained. Photoswitching is reversible, but finding just the right concept for the introduction of the reversible photoswitch in such a way that its photoisomerization dominates completely the activity to be regulated can be very difficult. The ideal photoswitch has probably not yet been found. Such an ideal photoswitch would operate without fatigue in water and could be fully switched to either one of the two photoisomers, which would have large differences in polarity, conductivity, or hinge properties. Despite the shortcomings in both areas, sophisticated experiments have already been designed, which we believe we have demonstrated. Clever combinations of techniques have just been developed in recent years—blurring the boundaries between these concepts—as we pointed out, for example, in Sections 2.1.2 and 2.1.3, when we discussed two-photon and wavelength-selective uncaging. The optogenetics approach is entirely different from the other two in so far as it relies not on chemical synthesis but instead on endogenously expressible systems. Its advantages are that optogenetic proteins can be addressed cell(-type) specifically, and that they rarely interfere with other cellular processes. However, optogenetic tools are generally not localized to specific cellular compartments, and thus the invoked signaling may be quite different from the equivalent signals mediated by endogenous, localized ion channels. As optogenetics are not restricted to ion channels or pumps and nature is extremely rich in light-sensitive proteins, this resource is just waiting to be harvested for novel optogenetic applications. The boundaries between optogenetics and the other two domains are also being blurred by approaches such as optochemical genetics. It will be very intriguing to see how many more bridging concepts will be developed in the future, such as, for example, in a recent study by Moore and coworkers, in which uncaging was realized "without light" deep inside an organism by Cherenkov radiation of 2-deoxy-2-[^{18}F]fluoro-D-glucose.[404] The big challenge in all three domains lies in their interdisciplinarity, as they rely on interactions between theoretical predictions, optics and spectroscopy, synthetic chemistry, biochemistry, biology, and even medicine. Only through close collaboration can the respective powers of each of these realms be harnessed. As this is difficult, only too often a brilliant idea comes to a standstill after the proof of principle study. It would be our hope that this Review can contribute to such a story being picked up again. We are convinced that there is a "bright future" ahead of us, with ever more intricate and subtle tools for studying (biological) processes.

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