Controlling Biological Function with Light

Hansen, Mickel Jens

Chapter 7
Wavelength-Selective Cleavage of Photoprotecting Groups: Strategies and Applications

Photocleavable protecting groups (PPGs) are extensively used in chemical and biological sciences. In their application, advantage is taken of using light as an external, non-invasive stimulus, which can be delivered with very high spatiotemporal precision. More recently, orthogonally addressing multiple PPGs, in a single system and with different wavelengths of light, has been explored. This approach allows one to independently control multiple functionalities in an external, non-invasive fashion. In this chapter, we discuss the design principles for dynamic systems involving wavelength-selective deprotection, focusing on the choice and optimization of PPGs, synthetic methods for their introduction and strategies for combining multiple PPGs into one system. Finally, we provide the reader with an instructive overview on how the wavelength-selective cleavage of photoprotecting groups can be applied in materials science, organic synthesis and biological systems.

This chapter was published as: **Wavelength-Selective Cleavage of Photoprotecting Groups: Strategies and Applications in Dynamic Systems.** M. J. Hansen, W. A. Velema, M. M. Lerch, W. Szymanski, B. L. Feringa, *Chem. Soc. Rev.* **2015**, *44*, 3358–3377.

7.1 Introduction

Many important chemical and biological systems consist of complex and highly dynamic networks of concurrent molecular processes. A major scientific challenge is to regulate such systems by controlling multiple processes simultaneously, as this will provide important tools for studying fundamental chemical and biological problems. To realize this, it is essential to apply external control elements that can be addressed without perturbing the system.[1–3] One appealing option, that has been explored excessively in recent years, is to use light as an external trigger and take advantage of its extraordinary properties: it can be delivered with high spatiotemporal precision, is non-invasive, does not leave sample contamination and its qualitative and quantitative properties can be precisely controlled.[2]

Two molecular approaches have been extensively investigated for controlling chemical and biological processes with light.[2,3] The first method relies on applying molecular photoswitches that can be reversibly isomerized between two or more states upon light irradiation. This isomerization results in an alteration in molecular properties, which in certain cases will be translated into a change in the chemical or biological effect.[2,4] However, these structural changes are often insufficient to exert a significant effect on the studied system. In such a situation the second approach, being the use of photocleavable-protecting groups (PPGs), is often a superior choice.[3,5]

The possibility to cage a functional group in a molecule with a PPG and liberate this functionality with the exceptional properties offered by light has caused PPGs to find a multitude of applications in organic synthesis,[6] material science [7] and biology.[8] Especially, the introduction of PPGs that can be uncaged with longer wavelengths of light made it possible to externally control biological processes with non-toxic and deep-tissue-penetrating visible light.[8] In order to regulate concurrent processes with light, it is crucial to be able to address these processes separately in an orthogonal and non-interfering fashion (Figure 1). This can be achieved by employing two or more PPGs with a difference in spectral properties like absorption maximum (λ_{max}), molar absorptivity (ε) and quantum yield (ϕ). The option to fine-tune the wavelength of deprotection offers great prospect to achieve high selectivity in controlling biomolecular processes.[9]

The idea of orthogonal deprotection has first been described in 2000 by Bochet and coworkers, where they explored the uncaging of two carboxylic acids that were protected with two different PPGs.[10] Since this seminal work, many systems have been described that are using wavelength-selective cleavage of PPGs to externally control multiple processes in parallel, applying light.[11–15] The terms ‘orthogonality’ and ‘wavelength selectivity’ are often used interchangeably in the literature to describe a situation where one PPG can be cleaved with certain selectivity over a second PPG, whereas orthogonality is defined as “a set of completely independent classes of protecting groups [...] in such a system, each class of protecting groups can

be removed in any order and in the presence of all other classes"[16] and as this has not been reported so far in the case of PPGs, we choose to only use the term 'wavelength selectivity'.

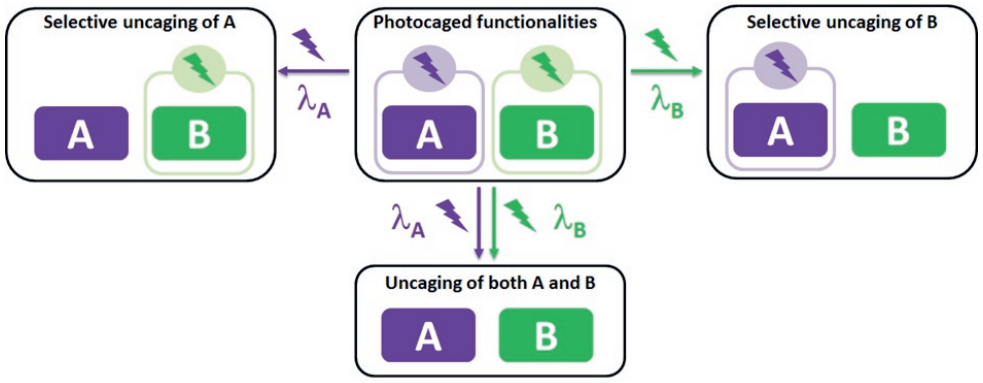

Figure 1. Schematic representation of orthogonal uncaging of two functionalities. Exposure to λ_A will selectively liberate functionality A, while irradiation with λ_B will selectively uncage functionality B. Irradiation with λ_A and λ_B will liberate both functionality A and B.

In this chapter, we will focus on the considerations for the development of wavelength-selective systems with multiple functional levels and we will provide guidelines for designing such systems. In the last part of this chapter, illustrative examples from recent literature will be introduced to present the broad scope of complex systems that can be studied and controlled using wavelength-selectively caged compounds. These examples show that wavelength-selective control over multiple concurrent processes can be a powerful tool in a variety of applications and it demonstrates the exciting prospects for the future.

7.2 Photo-cleavable Protecting Groups

The first reports of photocleavable protecting groups already date from the late 1960s with seminal publications from both the Schofield and Woodward groups.[17,18] Nowadays, a plethora of photo-cleavable protecting groups is available.[3-5] However, it is not trivial that all these protecting groups can be used for wavelength-selective deprotection. Therefore, in this section an overview of the most widely used photocleavable groups applicable for wavelength-selective deprotection will be given. Moreover, the focus will be on different ways to optimize photocleavable groups in such a way that they are suitable for wavelength-selective cleavage. This will mainly be done by an elaborate overview of substituent effects on the different classes of protecting groups (see Figures 2, 3 and 5 for; o-nitrobenzyl, coumarin and other derivatives).

For the application in wavelength-selective cleavage multiple requirements can be identified (*vide infra*, see section 7.3). A distinct and narrow absorption maximum is one of the requirements, besides high quantum yield and suppressed intra/intermolecular energy transfer, which are crucial to make protecting groups applicable in wavelength-selective cleavage. If this requirement is attained, protecting groups from different classes can be combined. As shown in Figure 5 (*vide infra*), in this way a large difference between absorption maxima can be obtained. With this in hand, combinations of protecting groups can be chosen for the design of systems with multiple functional levels.[7,12,19] Furthermore, even between two derivatives from the same class (see Figure 2), a small change in substituent or substitution pattern can lead to a large shift in λ_{max}. Therefore, strong substituent effects, shifting the absorption maxima away from each other, might allow the use of multiple protecting groups in one system.

One of the most extensively studied classes of photocleavable protecting groups are the *ortho*-nitrobenzyl derivatives.[5,20] Their relatively simple and divers structures which are readily available, and convenient attachment to the target molecules possessing various functionalities renders them highly useful for biological, as well as material, applications (*vide infra,* see section 7.5). As depicted in Figure 2, a wide variety of derivatives has been used for the application in wavelength-selective deprotection.

Substituent effects are not only of crucial importance for the absorption spectrum of the chromophore, but are also prominent for the stability of the C-R bond (where R is the caged group). By only making minor changes in the substitution pattern of structural derivatives of the *ortho*-nitrobenzyl protecting group it is possible to create PPGs that can be photocleaved with different wavelengths of light ($\lambda_{deprotect}$: 345-420 nm).[6,7,12–14,19,21–26] The most useful way to obtain a bathochromic (red) shift of the absorption band is the addition of an electron-withdrawing group (EWG) at the *para*-position. Additionally, substituting the *ortho*-nitrobenzyl core with a moderately electron-donating group (EDG) (-OR) in the *meta*-position permits cleavage with longer wavelength of light.[12–14,23,25] A significant hypsochromic shift, can be obtained by changing the α-substituent with respect to the R-group.[21,23,25] Furthermore, extending the linker between the chromophore and the cleavable C-R bond gives similar results.[12,22]

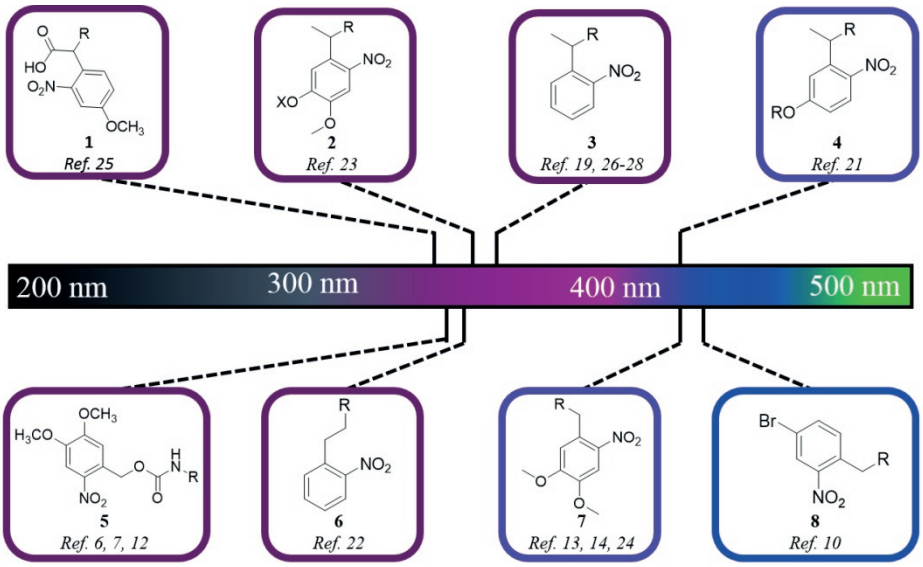

Figure 2. *Ortho*-nitrobenzyl derivatives used in wavelength-selective systems showing the diversity in protecting groups from one class available for these applications.

Besides *ortho*-nitrobenzyl derivatives, another widely applied class of photocleavable protecting groups comprise coumarin derivatives with general structures as depicted in Figure 3. Similarly to *ortho*-nitrobenzyl groups, coumarin protection offers certain advantages, including the easy synthesis and high biocompatibility.[5] Moreover, a range of coumarin derivatives are available for an extensive variety of deprotection wavelengths (see Figure 3).[7,9,15,19,25,27–31] Again, it can be noted that very small structural changes lead to large changes in deprotection wavelength ($\lambda_{deprotect}$: 312-505 nm). As depicted in Figure 3, electron-donating substituents in conjugation with the carbonyl or thio-carbonyl group, especially at the 7-position (see **10** for numbering), cause a bathochromic shift in absorption. Changing from the most widely used 7-diethylaminocoumarin towards a slightly less electron-donating 7-di(carboxy-methyl)amino group[22] led to a hypsochromic shift whereas altering it towards a 7-alkoxycoumarin[31] leads to an even larger shift in absorption. Not only is the maximum absorption band of great importance but in order to achieve high selectivity, also a small full width at half maximum (FWHM) is essential. From a recent report by Ellis-Davies and coworkers[9], as described below, it became apparent that the substituent at the 3-position is of crucial importance to obtain a well-defined absorption band at the λ_{max} without any 'shoulder' formation at lower wavelengths.

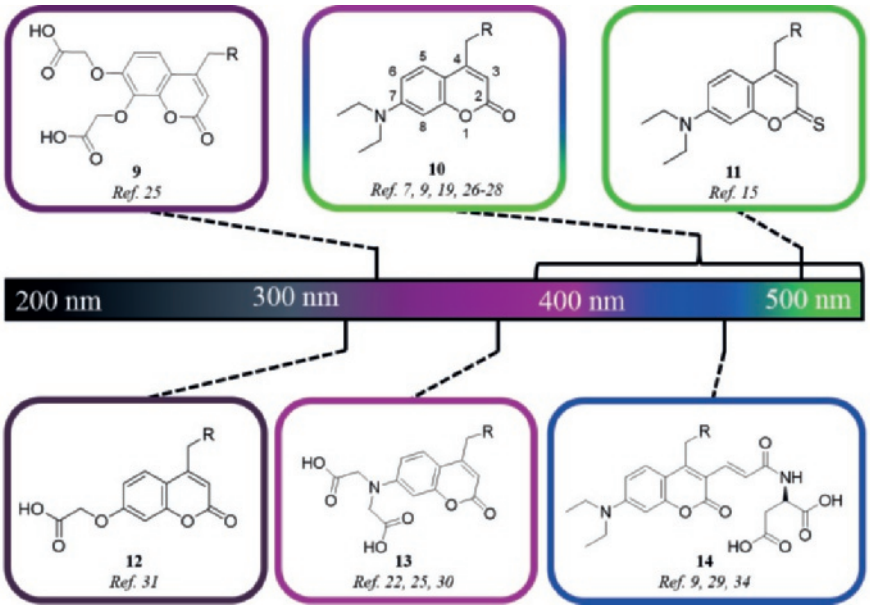

Figure 3. Coumarin derivatives showing substituent effects on the absorption maximum.

This seminal publication from the Ellis-Davies group[9] clearly illustrated the process of designing PPGs for the use in wavelength-selective uncaging. Starting with diethylaminocoumarin **10**, a systematic survey was performed, to develop a novel protecting group whose absorption maximum was red-shifted. An electron withdrawing nitro-group was added at the 3-position of the diethylaminocoumarin (**18**), which led to a large (>80 nm) bathochromic shift in the absorption maximum.[9] However, subsequent addition of a methyl substituent at the 4-position (**16**), which is desirable as a handle for the attachment to target molecules, shifted the absorption maximum back towards a lower wavelength (Figure 4).

This perturbation of the optimal features was attributed to a steric clash between the nitro and methyl groups, causing a twist in the overall coumarin molecule. To avoid steric clash, the authors decided to replace the nitro group for a 'smaller' cyano group (**17**),[9] which did not change the absorption maximum but lead to lowering of the absorption at the 300-350 nm range. To obtain a chromophore which could be used as a caging group, a glutamate derivative **15**, with a cyanophenyl instead of a cyano group at the 3-position, was synthesized. From this observation, it can be concluded that the substituents at the 3-position of the amino-coumarin should preferably be small but electron-withdrawing to decrease absorption in the 300-350 nm range. The low absorbance in this region is desirable because, as evident from Figures 2, 3 and 5, a variety of protecting groups showing photocleavage around 300-350 nm is available.

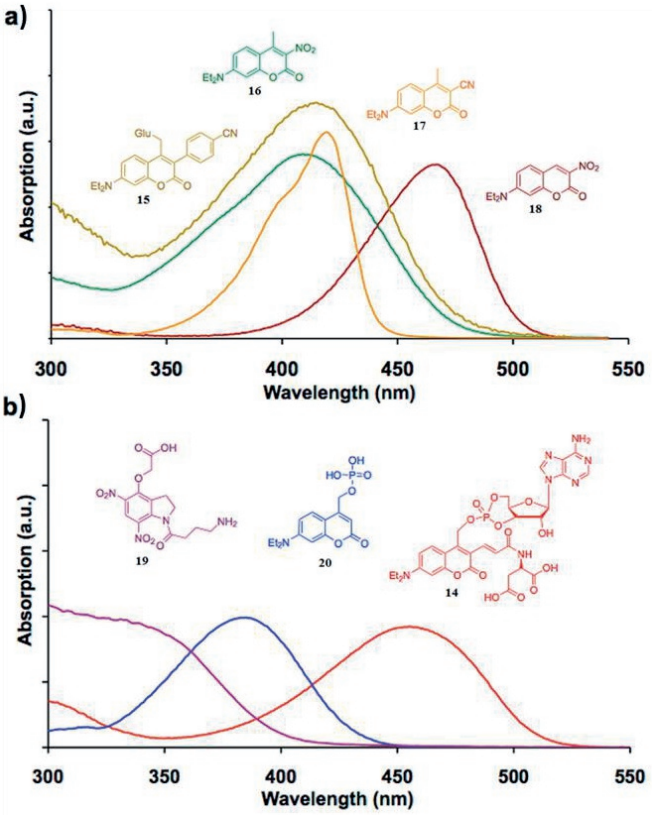

Figure 4. Towards the design of an optimized high-wavelength cleavable protecting group.[9] a) UV-vis spectra of the various coumarin derivatives obtained by systematic modification of a diethylaminocoumarin towards a glutamate-coumarin derivative. b) The specific absorption spectra for CDNI (**19**)-GABA, the original DEAC Pi (**20**) and the optimized DEAC450 (**14**) cAMP. Adapted with permission from Ref. 9. Copyright © 2013, American Chemical Society.

For example, combining the proposed DEAC450 protecting group **14** with an *ortho*-nitrobenzyl derivative (λ_{max} = 320 nm) as depicted in Figure 2, might lead to an orthogonal pair of protecting groups with high tolerance towards different functionalities. Finally, an amino-coumarin **14** (Figure 2) with a glutamate moiety at the 3-position, to enhance both the electronic properties and solubility, and a phosphate moiety at the 4-position, to couple to cAMP, was synthesized. A comparison between DEAC450 cAMP **14** (Figure 2) and CDNI-protected GABA **19** (Figure 4) showed UV-vis spectra (see Figure 4), which persuaded a near-optimal difference in absorption maxima and minima, allowing bidirectional modulation of neuronal firing rates in rat brain slices.[9]

Another elegant approach towards optimized coumarin protecting groups was reported in 2013 by Jullien, validating a novel thiocoumarin derivative for deprotection with blue-light.[15] Replacement of only the carbonyl group with a thiocarbonyl group in the original diethylaminocoumarin (see Figure 3; **10-11**) led to a significant red-shift in absorption. This bathochromic shift can be explained by the decrease in electronegativity, caused by the exchange of oxygen for sulfur, with concurrent increase of polarizability. Next to the *ortho*-nitrobenzyl and coumarin derivatives a multitude of different protecting groups are available for the use in wavelength-selective systems. As depicted in Figure 5, the groups used for wavelength selective cleavage range from simple carbonyl protecting salicylalcohols to inorganic ruthenium complexes.[6,9,14,19,24,26,29,30,32–34]

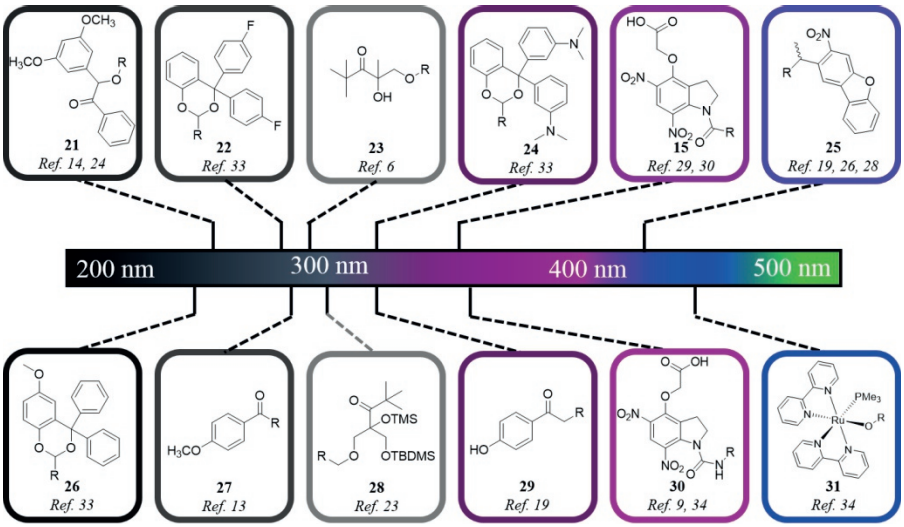

Figure 5. Representation of the different protecting groups with an absorption maximum between 220 and 450 nm.

Another example of the early work on substituent effects of PPGs, was reported by Wang *et al.* in 2011[33] where the structure of well-known salicyl alcohol protecting groups was modified (Figure 6). Salicyl alcohols are widely applied for the protection of carbonyl groups. In this study the possibility to both change the α-substituent and expand the aromatic chromophore were investigated. Expansion of the aromatic chromophore from a benzylic to a naphthalene backbone led to a bathochromic (red) shift, however, no acceleration of photocleavage at higher wavelengths was observed. This immediately rises another important issue when designing photo-cleavable groups, that is to always combine absorption spectra with data on quantum yields and/or absorptivities. Illustrative in this regard is also a study towards optimized α-substituents.[33] Starting from acetal **33**, which showed an absorption maximum around 297 nm, characteristic for the 5-methoxysalicyl chromophore, multiple novel acetals with higher wavelength absorption bands were

designed. For example, an acetal **24** with two 3-(dimethylamino)phenyl α-groups was presented that showed a red-shifted absorption around 309 nm in the UV-vis spectrum. The obtained absorption at higher wavelengths is desirable because, as stated before, by using combinations of red-shifted PPGs with PPGs which absorb in the 200-300 nm range, orthogonal systems can be designed. However, a disadvantage of the compounds in this study is the remaining absorption peak at 260 nm which still limits the application for wavelength-selective cleavage.

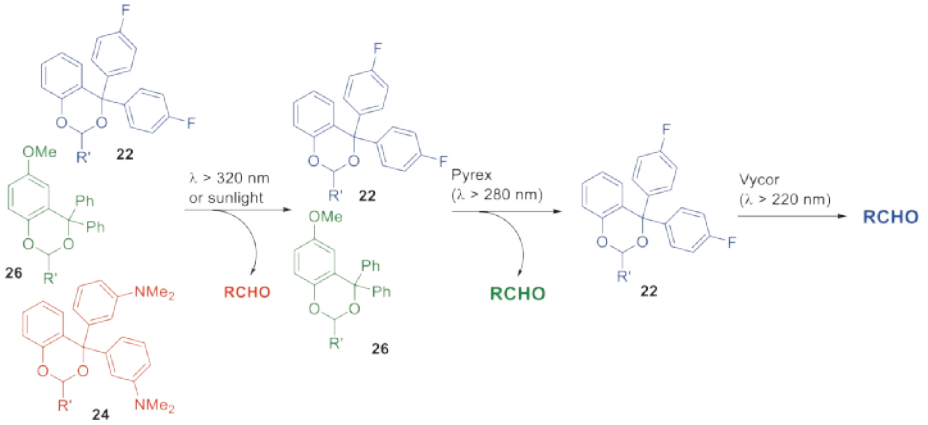

Figure 6. Design of a wavelength-selective system by Wang *et al.*, using salicyl alcohol derivatives altered by substituent effects. The different substituted salicyl-alcohol derivatives can be photocleaved at 320 nm, 280 nm, and 220 nm, respectively, allowing sequential deprotection of three different aldehydes. Adapted with permission from Ref. 33. Copyright © 2011, American Chemical Society

As mentioned before, not only the maximum absorption is of importance but also the efficiency of photocleavage is of great value in the design of novel PPGs. This was again nicely illustrated by showing that even though the absorption maxima of the different acetals (**22**, **24**, and **26**) were overlapping, still a difference in rate of cleavage could be observed because the α-substituents notably facilitated the benzylic C-O bond breakage. Using this method a wavelength selective system with a combination of the discussed photocleavable salicyl derivatives (**22, 26, 24;** Figure 6) was used to subsequently uncage carbonyl functionalities at 320, 280 and 220 nm, respectively (Figure 6).

However, a drawback of the work presented in this publication and multiple other reports is the need for a fixed sequence of deprotection (*vide infra,* see section 7.3 and 7.5). Moreover, the use of salicyl derivatives limits the applicability to protection/deprotection of carbonyl compounds.

The systematic surveys,[9,15,33] reported so far, showed that, by changing the substitution patterns, large shifts in absorption maxima and large changes in rate of photocleavage of different PPGs can be attained which allow the application of the

resulting groups for wavelength-selective uncaging. Moreover, it has been shown that a multitude of protecting groups, e.g. *ortho*-nitrobenzyl, coumarin and other derivatives, are available which might nicely syndicate in combinations suitable for wavelength-selective deprotection. This also exemplifies the high potential of future wavelength-selective systems to mimic complex systems in both biological as well as material sciences.

7.3 Design of Orthogonal Systems with Multiple Functional Levels

In the design of wavelength-selective uncaging systems, advantage may be taken of the difference in either of a number of properties between the components of the system. For instance, the first order reaction rate constant (k) of the PPG deprotection in compound (i), under the irradiation with light of a given wavelength, is directly correlated to the molar extinction coefficient at a that wavelength (ε) and the quantum yield of the process (ϕ) (formula 1).[35]

Formula 1: $k_i \propto \varepsilon_i \cdot \phi_i$

So far most of the systems described in the literature rely on exploiting the difference in absorbance (ε) at a given wavelength, which is usually much more pronounced than the difference in quantum yield (ϕ)[26,28]

In the ideal case, the two photocaged compounds (**A** and **B**, Figure 7a) have UV-vis spectra that do not overlap, *i.e.* for each of them it is possible to choose a wavelength of irradiation at which the extinction coefficient of the other one is near zero. In such a case, perfect orthogonality could be reached, meaning that each of the compounds could be uncaged in the presence of the other (Figure 7b and 7c). Such situations are, however, extremely rare. A representative photo-orthogonal system was reported by Scott *et al.*[36] for photoinitiation and photoinhibition of polymerization in lithography, however, in this case, no use of PPGs has been made.

It is common that, the spectra of different protecting groups partially overlap, as presented in Figure 7d. This mainly stems from the fact that most of the compounds, caged with visible light-sensitive protecting groups also show substantial absorbance in the UV-region. Therefore, photodeprotection of the UV-sensitive protecting groups will also cause partial deprotection of the other, visible light-sensitive, protecting group. In most cases, it is then possible to uncage them selectively only in a given sequence (Figure 7f), *i.e.* starting by irradiation at a longer wavelength (λ_B, Figure 7d), where compound A does not absorb ($\varepsilon_A \sim 0$) and subsequently, when **B** is fully uncaged, following by irradiation with shorter wavelength (λ_A) to uncage **A**. This application has been presented for several pairs of PPGs.[19,22,25,27,37]

While the spectral overlap does not allow full, sequence-independent orthogonality (Figure 7e), it is still possible to exploit the difference in kinetics of uncaging that

may prove large enough for the intended purpose, especially since full uncaging is not always needed.[7,28,38] The use of different wavelengths to select an optimal wavelength that would provide the most favorable ratio of uncaging has been reported as a way to improve the system, since the irradiation at the λ_{max} does not necessarily lead to optimal selectivity.[12,19,28] In a seminal paper, del Campo, Bochet and co-workers investigated the limits of the functional levels that may be reached using wavelength-selective uncaging of PPGs immobilized on a quartz surface.[12] By carefully choosing the wavelength of irradiation, they were able to define sets of PPGs (up to four in one set) that can be addressed selectively in one system, using sequential uncaging. Furthermore, they defined several pairs of PPGs that can be uncaged in a near-orthogonal fashion.

Much effort has been devoted to developing PPGs that would allow for fully orthogonal (not sequential) uncaging (*vide supra*, see section 7.2), with important contributions from the groups of Ellis-Davies,[9,39] Jullien,[15] Bochet[40] and Hagen[25]. The success of these projects underlines the potential of the approach based on exploiting the difference in absorption properties of PPGs at a given wavelength (ε, Formula 1) for the wavelength-selective activation. Main scientific targets in this context are the development of PPGs with a narrow wavelength range for deprotection and minor absorption outside of this range. Another important issue is to avoid the possibility of energy transfer between the PPGs. Furthermore, the quantum yield is of great importance: long irradiation times with UV or high-intensity light is undesirable, because it limits the applicability for bio- and material sciences. Therefore, it has to be kept in mind that the light intensity has to be compatible with the targeted system, because high intensity light might also cleave other chemical bonds (photodegradation) or influence biological functioning. Moreover, to allow for the use in bio-systems, photocleavable groups should be chemically stable and preferentially soluble in aqueous media, with the uncaging products showing negligible toxicity.

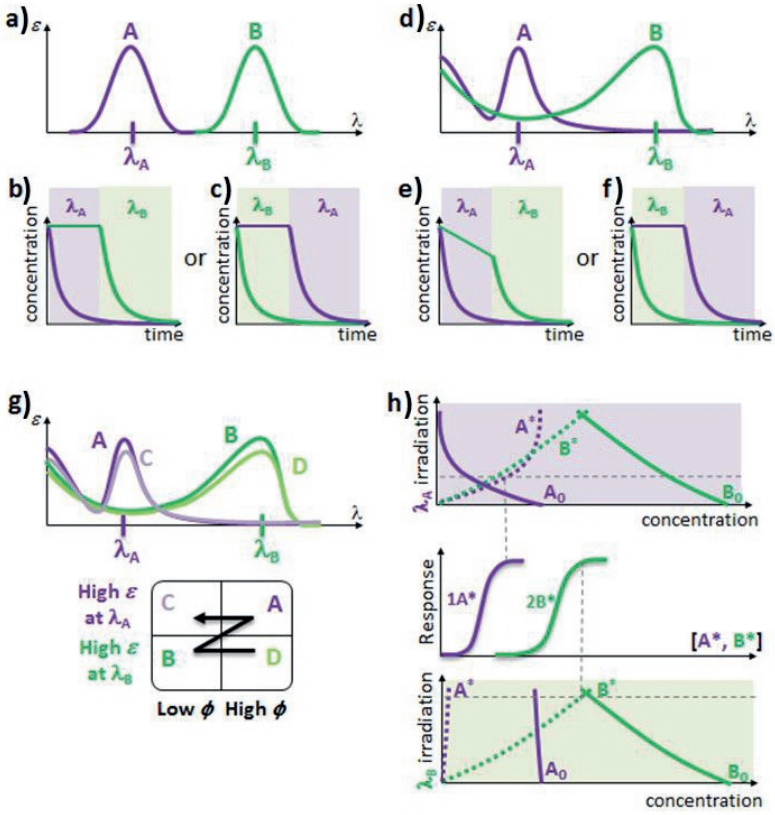

Figure 7. Strategies used in designing wavelength-selective uncaging systems, composed of two photocaged compounds **A** and **B** (ε = molar extinction coefficient, ϕ = quantum yield). a) Ideal situation, where the spectra of **A** and **B** do not overlap, which enables orthogonal deprotection irrespective of the sequence of wavelengths used (b, c). d) In a more realistic situation, the spectra of **A** and **B** overlap and selective deprotection is only possible when a correct sequence is used (f), i.e. first irradiation with light of longer wavelength, followed by light of shorter wavelength. In the reverse case, only partial selectivity can be achieved (e). g) With strong spectral overlap, high selectivity in sequential deprotection is still possible if the components of the system differ strongly in the quantum yield ϕ, as presented recently by Heckel *et al.*[41]) When compounds **A*** and **B*** (products of the uncaging of **A** and **B**, with starting concentrations of $\mathbf{A_0}$ and $\mathbf{B_0}$, respectively) are used for orthogonally controlling a system with a non-linear dose-response curve, even imperfectly selective uncaging can be translated to a fully orthogonal effect (see text for more details).

The development of new PPGs has been taken further with the use of two-photon uncaging groups **13** and **14** (Figure 3) by Ellis-Davies and co-workers.[29,30,34] Despite requiring stronger light sources, the two-photon process allows for both deep-

penetration and extremely high spatial resolution in biological tissues.[11] In the PPG context, mainly nitroindolinyl and nitrobenzyl groups have been used for two-photon uncaging, with wavelengths of light between 710 and 740 nm. Combination of these PPGs with **13** (uncaged at 830 nm)[30] or **14** (uncaged at 900 nm)[34] allows for increased selectivity.

So far, much less attention has been paid to using the differences in quantum yield (ϕ, Formula 1) for the design of photo-orthogonal systems. In principle, if sufficiently large differences in ϕ could be obtained, then even strong overlap of spectra would not prevent highly selective sequential uncaging (Figure 7f). Recently, the group of Heckel has presented a study on a mixture of four photo-protected nucleotides).[41] Two PPGs were used, connected to nucleic bases either by an oxygen or nitrogen atom. The nature of such a connection was shown to have a strong influence on the quantum yield of deprotection. This enabled to selectively uncage compound **D** with light of a longer wavelength (Figure 7g), in the presence of compound **A**-**C**, taking advantage of both the difference in extinction coefficients (**D** vs. **A**&**C**) and quantum yields (**D** vs. **B**). Afterwards, compound **B** could be uncaged through longer irradiation at the same wavelength. Subsequently, the difference in quantum yields was again used to selective uncage **A** in the presence of **C**. As a result, four levels of uncaging were obtained using only two PPGs and two wavelengths of light, which highlights the potential of exploiting differences in both quantum yields and absorption bands. Finally, the orthogonally photoprotected compounds are often employed to evoke, upon uncaging, downstream effects in the studied system. In many biological applications, dose-response curves have a non-linear character (Figure 7h, middle panel). This non-linearity can serve as an advantage in the design of the system, since imperfect selectivity in deprotection can still be translated to perfect orthogonality, if the starting concentrations are chosen properly, as exemplified by our recent work on the use of two photoprotected antibiotics for wavelength-dependent bacterial selection.[31] If one considers a mixture of two bacterial strains (**1** and **2**, Figure 7h) and two photocaged antibiotics (**A** and **B**, Figure 7h) which are chosen in such a way that antibiotic **A*** (product of uncaging of **A**) shows strong bactericidal activity on **1**, and antibiotic **B*** (product of uncaging of **B**) shows weaker bactericidal activity on **2** (with no cross-activities), it is possible to completely control which bacterial strain will be killed and which will grow. This can be achieved by using different starting concentrations ($\mathbf{A}_0 \neq \mathbf{B}_0$): $\mathbf{A}_0$ for photoprotected antibiotic **A** (UV-cleavable, Figure 7d) and $\mathbf{B}_0$ for photoprotected antibiotic **B** (UV-vis cleavable, Figure 7d). Irradiation with visible light (Figure 7h, lower panel) will result in much faster uncaging of **B** and, when the concentration of **B*** is reached that is sufficient to kill all bacteria **2** (Figure 7h, lower panel, dashed line), only very little **A*** will be present, insufficient to evoke a significant biological effect. Light of shorter wavelength results in much less difference in the kinetics of uncaging between **A** and **B** (Figure 7h, upper panel). However, due to very strong activity of **A*** on **1**, only very short irradiation is needed (Figure 7h, upper panel, dashed line), which is insufficient to uncage enough **B*** to kill bacteria **2**. Therefore,

weak selectivity in uncaging (Figure 7d) can be translated to completely orthogonal biological effects irrespective of the irradiation sequence.

In summary, there are several strategies that can be used for installing orthogonality into chromatically-selective uncaging systems. Of great value are studies on the development of new PPGs,[9,15,25,39,40] especially coupled with two-photon uncaging methods.[29,30,34] With the recent report from the group of Heckel,[41] it can be expected that more advantage will be taken not only from separating the absorption bands of PPGs, but also by employing the differences in quantum yields of deprotection. Finally, one always has to consider the final application. For example, the highly dynamic nature of biological systems and the non-linearity of biological response curves allows for the use of "non-perfect" systems to perform very well. Furthermore, different time-scales in deprotection might be beneficial for specific applications in biological systems, where the timing of trigger-events is crucial. Applying the basic principles of wavelength-selective deprotection to more complicated and dynamic systems (as for example in biology) offers great potential. However it also comes along with additional system-specific constraints that have to be taken into consideration. Such constraints are highly specific for the studied system. Nevertheless, general considerations can be summarized as follows:

1) The light-exposure must be adjusted so that it does not show any effect, either negative (toxic/damaging) or positive, *e.g.* on biological samples;

2) The released part of the uncaging group must not show toxicity, *e.g.* to biological samples (e.g. when highly reactive aldehydes are generated).

3) The solubility of the caged and uncaged bioactive molecule can differ remarkably.

4) The kinetics and behavior of the light-driven uncaging process is highly solvent (buffer) dependent.

7.4 Synthetic Considerations

The synthetic procedures towards most of the well-established PPG precursors have been reviewed elsewhere.[5] The synthesis of the precursors for the recently-introduced PPGs that are most frequently used in multi-PPG, wavelength-selective release systems, has been recently published for the following protecting groups: **14**[9,29,34], **21**[14,24], **10**[7,9,19,27,28], **11**[15], **13**[25,31], **1**[25] and **9**.[25] The general strategies for the introduction of PPGs onto most important reactive groups (alcohols, thiols, amines, carboxylic acids and phosphates) are summarized in Figure 8.

Most frequently, PPGs possess a nucleophilic group, which is most often a hydroxyl, although in some cases a secondary amine (indolinyl-based PPGs **19, 30**) is present. These functionalities are used for the photoprotection of target molecules (Figure 8),

in general by conversion to a leaving group (via introduction of a halide or pseudohalide) to activate them for a reaction with a nucleophile.[9,25]

Protection of alcohols and thiols (Figure 8a) is usually achieved using a carbonate[12] or ether[25] linker. Amines can be protected by forming a carbamate linker with the PPG, introduced through the reaction of the PPG-hydroxyl group with an isocyanate [7,12] or by converting the PPG-hydroxyl into an activated carbonate/carbamate and reaction with the target amine [7,10,15] (Figure 8b). Alternatively, the indolinyl-based PPGs were transformed into chloroformamides and reacted with target amines to form photocleavable ureas [12,22] (Figure 8b).

Carboxylic acids can be protected with PPGs as photocleavable esters or amides (Figure 8c). The traditional ester formation methods are used, including Fisher esterification,[6] the reaction of PPG-hydroxyl groups with acyl chlorides/anhydrides[24,40] and the use of various coupling reagents to activate the carboxylic group for reaction with the PPG.[12,29,30,34,40] Also reactions of halide-bearing PPGs with carboxylic acids were used.[12] An alternative modular approach uses aldehyde-bearing PPGs, that react with acids and isocyanides in Passerini reactions, allowing for one-step introduction of a chosen photocleavable moiety and a tag, such as a photosensitizer.[37] Photocleavable amides were formed from indolinyl-based PPGs and acids using coupling reagents.[30]

Photoprotection of phosphates has been achieved by transforming the PPG-hydroxyl group into a leaving group.[9] Also, the construction of the photocleavable phosphate ester can be done in two steps, by first forming the *N*,*N*-diisopropylphosphoramidite and subsequent reaction with the chosen alcohol.[27]

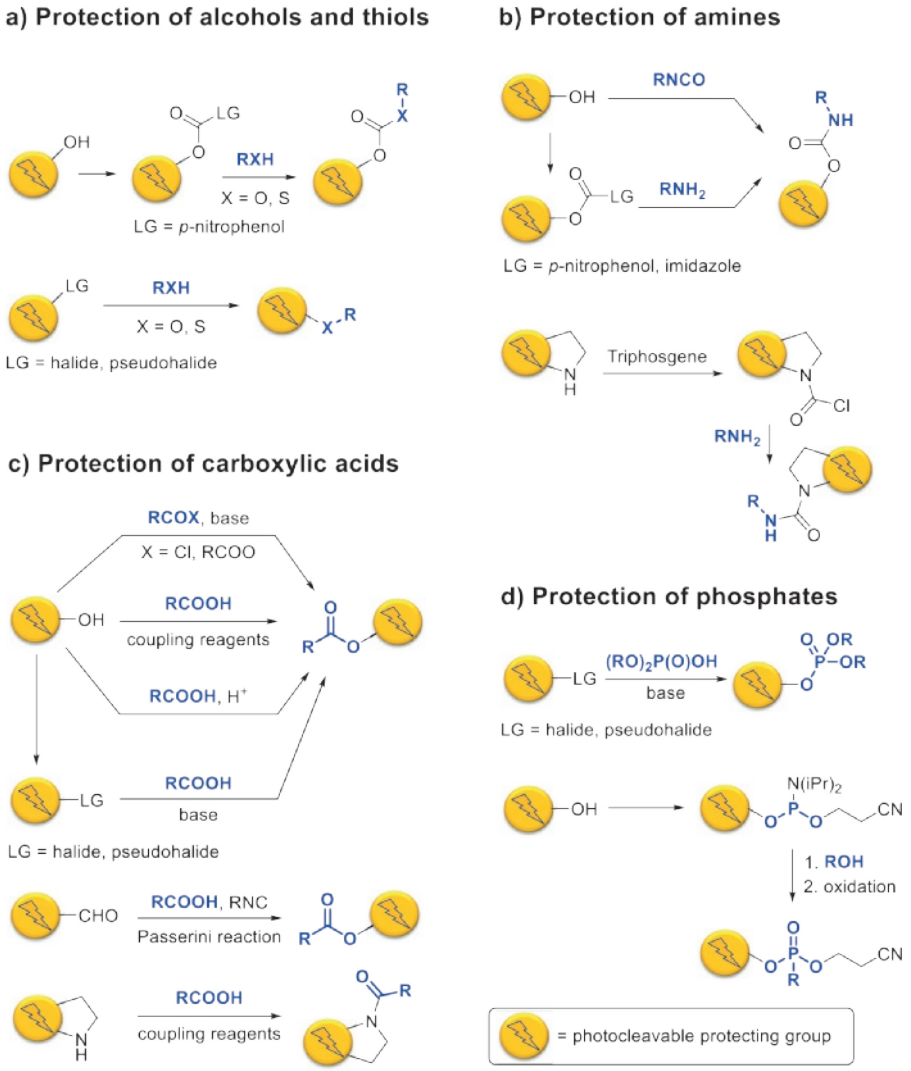

Figure 8. General strategies used for the introduction of PPGs onto reactive groups of a) thiols and alcohols, b) amines, c) carboxylic acids and d) phosphates.

7.5 Illustrative Examples and Applications

The following section aims at illustrating important findings and advances of this emerging field with specific applications. Furthermore, the concept of wavelength-selective uncaging will be presented in a broader context. The potential of selectively addressing multiple levels in one system is show-cased here with examples from a diverse set of disciplines ranging from organic synthesis and material sciences to

molecular biology and neurophysiology. To complement the design guidelines (*vide supra, see section 3*), instructive examples are highlighted that provide further insights on application-specific constraints that have to be taken into consideration for designing new systems. In seminal studies, showing the proof of concept, by Bochet and coworkers,[10,24] efforts had been directed towards the development of simple wavelength-selective PPG pairs, which were applied in both an inter- and intramolecular fashion. In both cases, a crucial and limiting factor for the development of such pairs was the loss of selectivity due to inter/intra-molecular energy transfer. Initial work focused on the use of 3,5-dimethoxybenzyl alcohol derivatives, that are cleavable with light of short wavelengths of $\lambda < 350$ nm and 2-nitroveratryl derivatives (see Figure 2) that are cleaved with longer wavelengths of $\lambda \geq 350$ nm). However, in this case, intermolecular energy transfer interfered with wavelength-selective cleavage. By using rational design, the lifetime of the excited state of the dimethoxybenzyl alcohol derivative was adjusted and the intermolecular energy transfer could be reduced, leading to high selectivities.

Notably, when applied to an intramolecular system, similar high selectivity could be achieved with the same PPG pair. Interestingly, effects of energy transfer would be expected to be most pronounced in an intramolecular setting. Furthermore, intramolecular energy transfer does directly depend on the distance between the potential donor and acceptor. However, very low energy transfer, and no significant distance dependence was observed, highlighting that adjusting the lifetime of the excited state of the dimethoxybenzyl alcohol derivative was crucial for the success of this experiment. In 2003, the concept of wavelength-selective PPGs was applied to organic synthesis, more specifically for solid-phase peptide synthesis (Figure 9).[6] The sequential chemical synthesis of biopolymers, like nucleic acids and peptides/proteins, requires the use of a multitude of orthogonal protecting groups. Cleavage of each protecting group needs to occur under mild conditions that do not interfere with other functionalities/protecting groups. Herein, light-control offers great advantage (*vide supra,* see section 7.1) due to its mildness and orthogonality to most other reaction conditions.

The Bochet group reported the synthesis of a pentapeptide (Leu-Enkephalin; H-Tyr-Gly-Gly-Phe-Leu-OH) in 55% overall yield *via* solid-phase peptide synthesis (SPPS) with PPGs and a photolabile linker. For the synthesis to be successful, a photolabile *tert*-butyl ketone linker **23** (Figure 5; cleavable at 335 nm) on a TentaGel-resin was used. Amino acids were *N*-protected with a 6-nitroveratryloxycarbonyl **7** (Figure 9) temporal protecting group (cleavable at 360 nm). Interestingly, the presence of the resin did not interfere with the photolytic process. Initial problems with *ortho*-nitrobenzyl-deprotection were encountered, but circumvented by using a scavenger (0.5% semicarbazide hydrochloride) to tap the aldehyde generated *in-situ* upon uncaging. In summary, the combination of wavelength-selective cleavage of the linker and a temporal protecting group allowed for very mild reaction conditions.

In a more recent report of a solution-phase peptide synthesis (see Figure 9b), this concept was taken a step further.[42] By performing both the coupling and the deprotection step photochemically, it was possible to synthesize a pentameric part of the osteogenic growth peptide (OGP(10-14), Ddz-Tyr(O^{t}Bu)-Gly-Phe-Gly-Gly-O^{t}Bu) without the need for additional reagents for coupling and deprotection. In order to succeed, these iterative steps needed to be addressed in a wavelength-selective fashion. *N*-acylated 5,7-dinitroindoline **33** (Figure 9) was chosen as a light-triggered activator group for the C-terminus of amino acids. This group renders the carboxy group inert towards nucleophilic attack, but allows for photoactivation of the amino acid towards acylation upon irradiation at 375 to 385 nm. Ddz **32** (Figure 9) was used for protection of the amino group. This particular protecting group **32** could be deprotected with a significant lower wavelength of irradiation (300 nm). Overall, this approach works relatively well with yields of 81-92% in the peptide couplings, using exclusively light for deprotection. However, this method is still hampered by long reaction times, the need for purification of intermediates and the laborious syntheses of building blocks.

Early research has mainly focused on the wavelength-selective cleavage of a pair of PPGs. Obviously, the use of wavelength-selective uncaging is not limited to simple pairs of PPGs. The number of functional levels that can be selectively addressed, which is defined by the number of distinct wavelength-selective deprotections possible for a given system, poses a limiting factor for the complexity of a possible application. Thus more recent efforts have been directed towards selectively addressing >2 functionalities in one system.[12,19,41]

Notably, the groups of Bochet and del Campo[12] showed that in 2011 up to four independent functional levels on a single surface could be selectively addressed with wavelengths ranging from 255 to 435 nm (Figure 10). Photoactivatable surfaces were generated, using seven different types of photoprotected surface-attached silanes (Figure 10a). The different PPGs were attached to amine-, thiol- or carboxylic acid-groups. The PPGs belonged to five different classes (Figure 10b): *p*-hydroxyphenacyl **27**, 7-nitroindoline **15**, (coumarin-4-yl) methyl **10**, benzoin **21** and *o*-nitrobenzyl **3, 7**. In solution, the coupling of the different PPGs to silanes did not substantially change the shape of the UV-absorption spectra and λ_{max} of the photocleavable groups (Figure 10c). Subsequently, a photoresponsive surface was obtained by reaction of the caged silanes with a silica surface. Interestingly, this did not alter the profile of the UV spectra, which suggests that no surface-induced variations of the photochemical properties were manifest. This was explained by the lack of interaction of the chromophores with the surface or between themselves. Importantly, by carefully screening different wavelengths, various functional levels could be addressed. It is important to note, however, that the reported selective combinations are in general not orthogonal and thus depend on the sequence of irradiation. Interestingly, in one particular case, when **10** and **21** were used, intensity-selectivity could be obtained by adjusting the energy dose of irradiation, while

keeping the wavelength constant, by taking advantage of a favorable combination of kinetics, extinction coefficients and quantum yield (*vide supra,* see section 7.3).

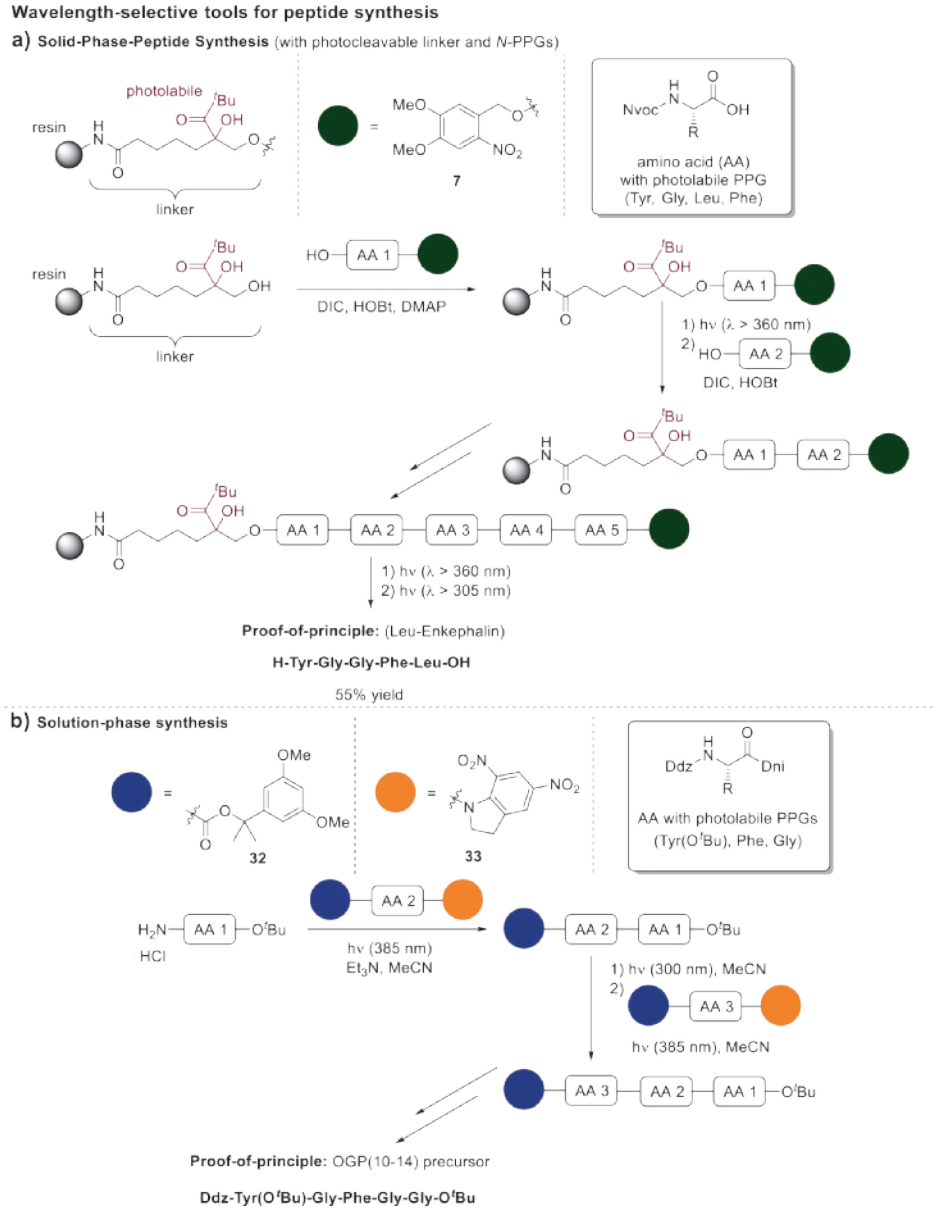

Figure 9. Highlighted examples of photocontrol in peptide synthesis by Bochet and co-workers.[6,42] a) Solid-Phase-Peptide Synthesis example and b) solution-phase example.

Adapted with permission from Refs. [6,42]. Copyright © 2003, American Chemical Society (Ref. 6). Copyright 2012 The Royal Society of Chemistry (Ref. 42).

7.6 Multiple Functional Levels

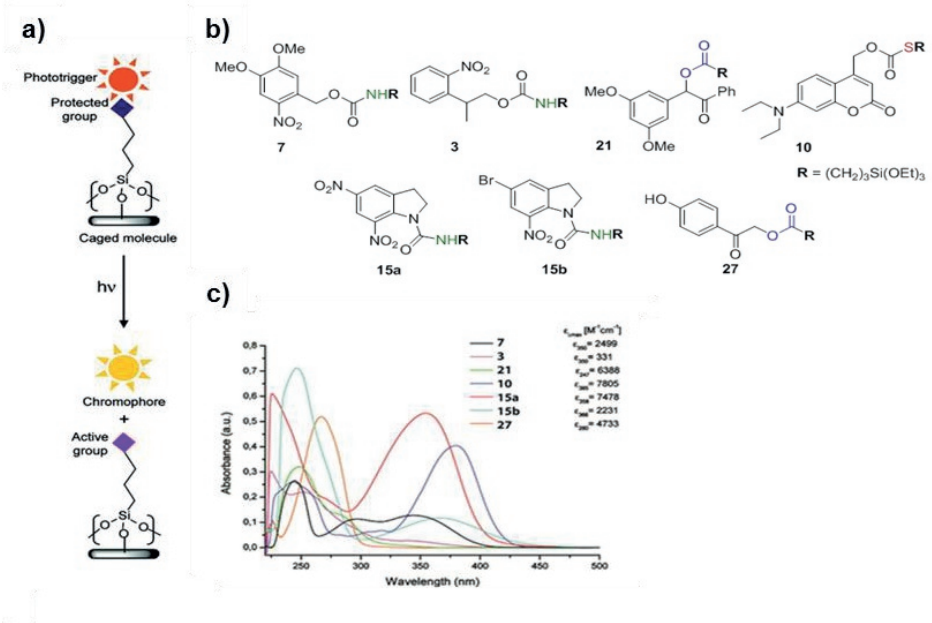

Figure 10. Addressing multiple independent functional levels. a) PPG caged silanes on a surface can be selectively cleaved using light. b) The seven different PPGs used for this study.[12] c) UV-vis absorption spectra of the protected silanes in solution. Adapted with permission from Ref. 12. Copyright © 2011, American Chemical Society.

Based on a simple analysis of the overlap of absorption spectra (Figure 10c), one would expect only limited selectivity. Especially the individual absorption maxima (λ_{max}) can be quite close to each other. However, when choosing wavelengths further away from the absorption maximum, better separation of the distinct wavelengths for deprotection was obtained, allowing for selective deprotection. The wavelength screening approach described in this report impressively lead to multiple levels of selective uncaging. The question arises if this way of designing multiple levels of functionality is the most desirable from a rational design point-of-view. By development and optimization of novel or improved protecting groups (*vide supra,* see section 7.2), higher selectivities with comparable deprotection-efficiencies might be obtained. Still, this work illustrated that not only the design and combinations of protecting groups is of importance but also that promising results can be obtained by carefully screening wavelengths of irradiation in order to obtain high selectivity.

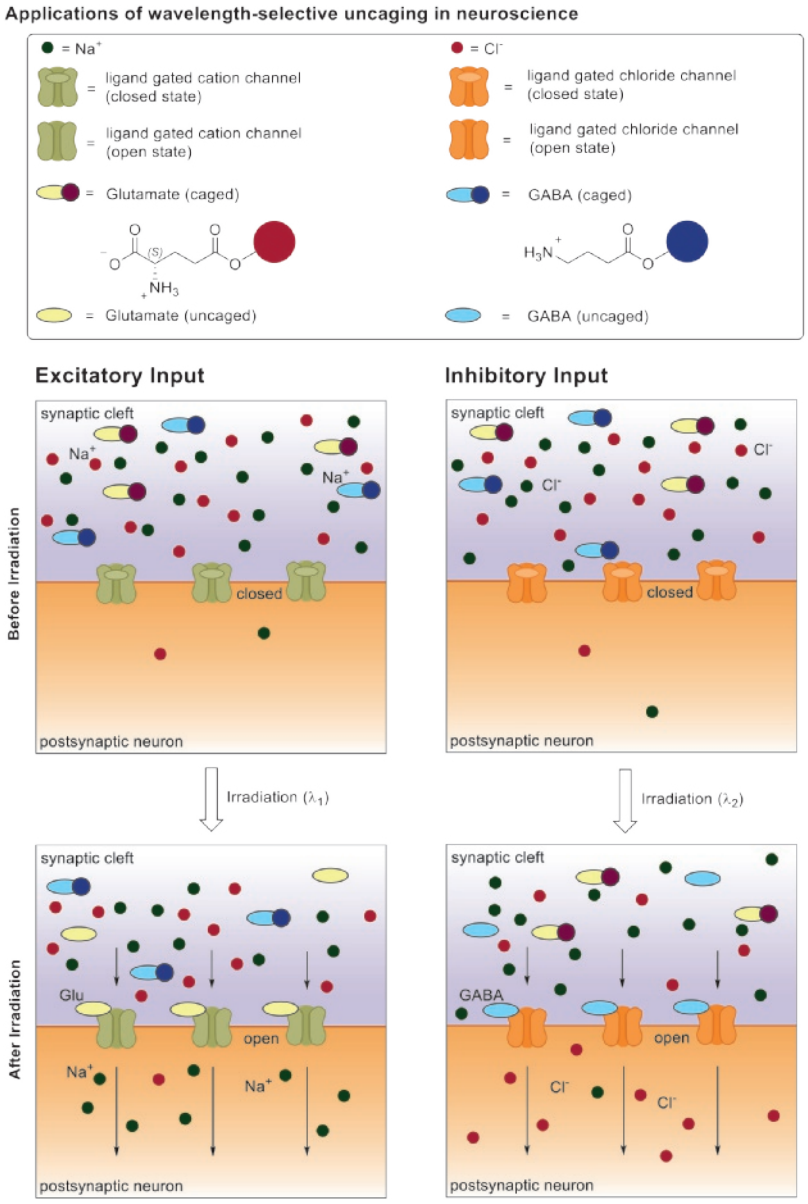

Figure 11. Wavelength-selective release of neurotransmitters for the study of integration of excitatory and inhibitory signals. a) Underlying principle for the experimental design. Irradiation with light (λ_1) leads to selective uncaging of the excitatory neurotransmitter Glu. Uncaged Glu then activates ligand-gated cation channels thus leading to an influx of e.g. Na^+, resulting in an excitatory stimulus. Similarly, irradiation with light (λ_2) leads to selective uncaging of the inhibitory

neurotransmitter GABA. Uncaged GABA then activates ligand-gated chloride channels that lead to an influx of Cl^-, resulting in an inhibitory stimulus.[13,44]

The previous example raises an important concern: the number of selectively addressable functional levels for wavelength-selective uncaging is inherently dependent on spectral properties, especially on the FWHM/line-width of the absorption bands (*vide supra,* see section 7.2 and 7.3). Therefore, only a limited set of functional levels is experimentally feasible. The kinetics of photodeprotection of a given PPG are however, not solely dependent on the spectral properties and can be chemically fine-tuned by the choice of appropriate substitutions/modifications of the PPG-core.[40] Importantly, the nature of the connecting atom can also have a very strong impact on the reactivity of the PPG.[43] It may thus be possible to address certain functional levels solely by making intelligent use of deprotection kinetics.

More than a decade after the first report of wavelength-selective deprotections, only a few examples of more than two functional levels have been reported, which exemplifies the difficulty to achieve suitable applications in such complex responsive systems. Biological systems show an astonishing level of complexity mainly caused by the dynamic interplay of single components and networks in combination with highly non-linear response curves for internal and external stimuli. Meaning it is not only important where and when something is activated/deactivated, but also how much something is activated/deactivated. In a biological setting, many existing methods to control or probe functions and effects are either too invasive or the level of temporal or spatial control is not sufficient. The use of light can provide a solution in certain cases.[3] From a design strategic point of view, using the concept of wavelength-selective control over function in challenging systems brings additional system-specific constraints. Without limiting the applicability of the desired system, they have to be taken into consideration for successful application (*vide supra*, see section 7.3). A prominent example for the successful application of wavelength-selective uncaging is found in neuroscience, specifically the study of integration and logical gate systems for single synapses and neurons with excitatory and inhibitory neurotransmitters. This field of research has recently been summarized in an excellent review.[44] (*S*)-glutamic acid (Glu, excitatory) and γ-amino butyric acid (GABA, inhibitory) is a commonly used pair to study neuronal integration mechanisms (Figure 11). Traditional approaches have made use of the presynaptic release of neurotransmitters to study this process.[11] The use of wavelength-selective PPGs offers highly spatio-temporal control of release of neurotransmitters for single synapses and parts of neurons, allowing for studying neurotransmitter distributions and integration of excitatory and inhibitory signals.

Importantly, in the quest to influence and control the complexity of biological systems one should not rely on single approaches and concepts, but should be open to combine different strategies. A remarkable report from Jullien and co-workers[15] from 2013 highlights this aspect: The development of a 7-diethylamino-4-thiocoumarinylmethyl based photolabile protecting group specifically tailored for

the wavelength region of 470-490 nm was combined with a visible light photoswitch to affect developmental processes in living zebrafish embryos. Using this PPG to cage a cyclofen-OH analogue, activation of a transcription factor (λ = 488 nm) was obtained, which lead to size reduction or loss of eye development. However, isomerization of 13-*cis*-retinoic acid into all-*trans*-retinoic acid (λ = 355 nm) led to the rescue of hindbrain formation, which was achieved by interference of all-*trans*-retinoic acid with a diethylaminobenzaldehyde inhibitor. This, when performed in zebrafish embryos, illustrated the possibility to combine activation upon *cis-trans* isomerization with activation upon photolysis, using a dissimilar wavelength, for the control of different biologically-active substrates *in vivo*.

7.7 Conclusions and Outlook

In this chapter, we have discussed the principles behind designing complex systems in which multiple functional levels can be independently addressed with light, using wavelength-selective removal of photocleavable protecting groups. Our aim was to provide the reader with guidelines on designing and choosing protecting groups, combining them towards maximum selectivity and incorporating them into target molecules. These considerations were then illustrated by selected examples in which the multiple possible applications for wavelength-selective uncaging in biology, synthetic chemistry and surface science are show-cased.

The way in which the multi-level systems are designed has been greatly influenced by the recent expansion of the PPG toolbox, and the increasing understanding of the influence of the PPGs molecular structure on their photochemical properties. The new developments in PPG chemistry were highlighted in this chapter. Furthermore, also other concepts were presented that can be used as design principles: the use of two-photon absorption processes[30] and the advantages that can be taken from the differences in PPG uncaging quantum yields.[41]In general, the design of the system in which multiple functions are to be addressed in a selective fashion depends on the number of functional levels needed. Of crucial importance is also if these levels can be addressed in a given sequence or if a complete, sequence-independent selectivity is required. If sequential deprotection is sufficient, sets of protecting groups have been proposed that allow for up to four different functions to be separately addressed,[12] providing that the deprotection is carried out starting from the longest wavelength and proceeding to irradiation with light of increasing energy. The complete, sequence-independent selectivity was shown so far for the uncaging of only two compounds in a mixture.[12]

In order to provide a useful tool for complex, dynamic systems, wavelength-selective uncaging not only needs to show high selectivity for several functional levels, but should also exhibit high flexibility in wavelength range. In general, the systematic understanding and development of a widely applicable and freely combinable set of wavelength-selective PPG libraries that can be easily adapted to the needs of

different applications cannot be overemphasized. Fortunately, impressive advances by several groups have already been made and continue to transform the field. In recent years, a growing number of reports highlight the great potential of wavelength-selective uncaging in more complicated and dynamic applications, ranging from chemical biology to responsive materials. The level of control, and especially its highly non-invasive application, offer great promise and give a hindsight of future possible developments, a journey that has just begun.

7.8 References

(1) Gautier, A.; Gauron, C.; Volovitch, M.; Bensimon, D.; Jullien, L.; Vriz, S. *Nat. Chem. Biol.* **2014**, *10*, 533–541.

(2) Szymański, W.; Beierle, J. M.; Kistemaker, H. A. V.; Velema, W. A.; Feringa, B. L. *Chem. Rev.* **2013**, *113*, 6114–6178.

(3) Brieke, C.; Rohrbach, F.; Gottschalk, A.; Mayer, G.; Heckel, A. *Angew. Chem., Int. Ed.* **2012**, *51*, 8446–8476.

(4) Velema, W. A.; Szymanski, W.; Feringa, B. L. *J. Am. Chem. Soc.* **2014**, *136*, 2178–2191.

(5) Klán, P.; Šolomek, T.; Bochet, C. G.; Blanc, A.; Givens, R.; Rubina, M.; Popik, V.; Kostikov, A.; Wirz, J. *Chem. Rev.* **2013**, *113*, 119–191.

(6) Kessler, M.; Glatthar, R.; Giese, B.; Bochet, C. G. *Org. Lett.* **2003**, 5, 1179–1181.

(7) Stegmaier, P.; Alonso, J. M.; Campo, A. Del. *Langmuir ACS J. surfaces colloids* **2008**, *24*, 11872–11879.

(8) Deiters, A. *ChemBioChem* **2010**, *11*, 47–53.

(9) Olson, J. P.; Banghart, M. R.; Sabatini, B. L.; Ellis-Davies, G. C. R. *J. Am. Chem. Soc.* **2013**, *135*, 15948–15954.

(10) Bochet, C. G. *Tetrahedron Lett.* **2000**, *41*, 6341–6346.

(11) Ellis-Davies, G. C. R. *Nat. Methods* **2007**, *4* (8), 619–628.

(12) San Miguel, V.; Bochet, C. G.; del Campo, A. *J. Am. Chem. Soc.* **2011**, *133*, 5380–5388.

(13) Stanton-Humphreys, M. N.; Taylor, R. D. T.; McDougall, C.; Hart, M. L.; Brown, C. T. A; Emptage, N. J.; Conway, S. J. *Chem. Commun.* **2012**, *48*, 657–659.

(14) Bochet, C. G. *Angew. Chem., Int. Ed.* **2001**, *40*, 2071–2073.

(15) Fournier, L.; Gauron, C.; Xu, L.; Aujard, I.; Le Saux, T.; Gagey-Eilstein, N.; Maurin, S.; Dubruille, S.; Baudin, J.-B.; Bensimon, D.; Volovitch, M.; Vriz, S.; Jullien, L. *ACS Chem. Biol.* **2013**, *8*, 1528–1536.

(16) Barany, G.; Merrifield, R. B. *J. Am. Chem. Soc.* **1977**, *99*, 7363–7365.

(17) Barltrop, J. A.; Plant, P. J.; Schofield, P. *Chem. Commun.* **1966**, 22, 822–823.

(18) Patchornik, A.; Amit, B.; Woodward, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 6333–6335.

(19) Rodrigues-Correia, A.; Weyel, X. M. M.; Heckel A. *Org. Lett.* **2013**, *15*, 5500–5503.

(20) Yu, H.; Li, J.; Wu, D.; Qiu, Z.; Zhang, Y. *Chem. Soc. Rev.* **2010**, *39*, 464–473.

(21) Griffin, D. R.; Kasko, A. M. *ACS Macro Lett.* **2012**, *1*, 1330–1334.

(22) Priestman, M. A.; Sun, L.; Lawrence, D. S. *ACS Chem. Biol.* **2011**, *6*, 377–384.

(23) Ladlow, M.; Legge, C. H.; Neudeck, T.; Pipe, A. J.; Sheppard, T.; Yang, L. L. *Chem. Commun.* **2003**, 16, 2048–2049.

(24) Blanc, A.; Bochet, C. G. *J. Org. Chem.* **2002**, *67*, 5567–5577.

(25) Kotzur, N.; Briand, B.; Beyermann, M.; Hagen, V. *J. Am. Chem. Soc.* **2009**, *131*, 16927–16931.

(26) Schäfer, F.; Joshi, K. B.; Fichte, M. a H.; Mack, T.; Wachtveitl, J.; Heckel, A. *Org. Lett.* **2011**, *13*, 1450–1453.

(27) Goguen, B. N.; Aemissegger, A.; Imperiali, B. *J. Am. Chem. Soc.* **2011**, *133*, 11038–11041.

(28) Menge, C.; Heckel, A. *Org. Lett.* **2011**, *13*, 4620–4623.

(29) Amatrudo, J. M.; Olson, J. P.; Lur, G.; Chiu, C. Q.; Higley, M. J.; Ellis-Davies, G. C. R. *ACS Chem. Neurosci.* **2014**, *5*, 64–70.

(30) Kantevari, S.; Matsuzaki, M.; Kanemoto, Y.; Kasai, H.; Ellis-Davies, G. C. R. *Nat. Methods* **2010**, *7*, 123–125.

(31) Velema, W. A.; van der Berg, J. P.; Szymanski, W.; Driessen, A. J. M.; Feringa, B. L. *ACS Chem. Biol.* **2014**, *9*, 1969–1974.

(32) Kaupmees, K.; Tolstoluzhsky, N.; Raja, S.; Rueping, M.; Leito, I. *Angew. Chem., Int. Ed.* **2013**, 52, 11569–11572.

(33) Yang, H.; Zhang, X.; Zhou, L.; Wang, P. *J. Org. Chem.* **2011**, *76*, 2040–2048.

(34) Olson, J. P.; Kwon, H.-B.; Takasaki, K. T.; Chiu, C. Q.; Higley, M. J.; Sabatini, B. L.; Ellis-Davies, G. C. R. *J. Am. Chem. Soc.* **2013**, *135*, 5954–5957.

(35) DeForest, C. A.; Anseth, K. S. *Nat. Chem.* **2011**, *3*, 925–931.

(36) Scott, T. F.; Kowalski, B. A.; Sullivan, A. C.; Bowman, C. N.; McLeod, R. R. *Science* **2009**, *324*, 913–917.

(37) Szymański, W.; Velema, W. A.; Feringa, B. L. *Angew. Chem., Int. Ed.* **2014**, *53*, 8682–8686.

(38) Azagarsamy, M. A.; Anseth, K. S. *Angew. Chem., Int. Ed.* **2013**, *52*, 13803–13807.

(39) Momotake, A.; Lindegger, N.; Niggli, E.; Barsotti, R. J.; Ellis-Davies, G. C. R. *Nat. Methods* **2006**, *3*, 35–40.

(40) Blanc, A.; Bochet, C. G. *Org. Lett.* **2007**, *9*, 2649–2651.

(41) Rodrigues-Correia, A.; Knapp-Bühle, D.; Engels, J. W.; Heckel, A. *Org. Lett.* **2014**, *16*, 5128–5131.

(42) Débieux, J.-L.; Bochet, C. G. *Chem. Sci.* **2012**, *3*, 405-406.

(43) Solomek, T.; Mercier, S.; Bally, T.; Bochet, C. G. *Photochem. Photobiol. Sci.* **2012**, *11*, 548–555.

(44) Amatrudo, J. M.; Olson, J. P.; Agarwal, H. K.; Ellis-Davies, G. C. R. *Eur. J. Neurosci.* **2015**, *41*, 5–16.