DOI: 10.1002/anie.201204203

Two-Photon Probes

From One-Photon to Two-Photon Probes: “Caged” Compounds, Actuators, and Photoswitches

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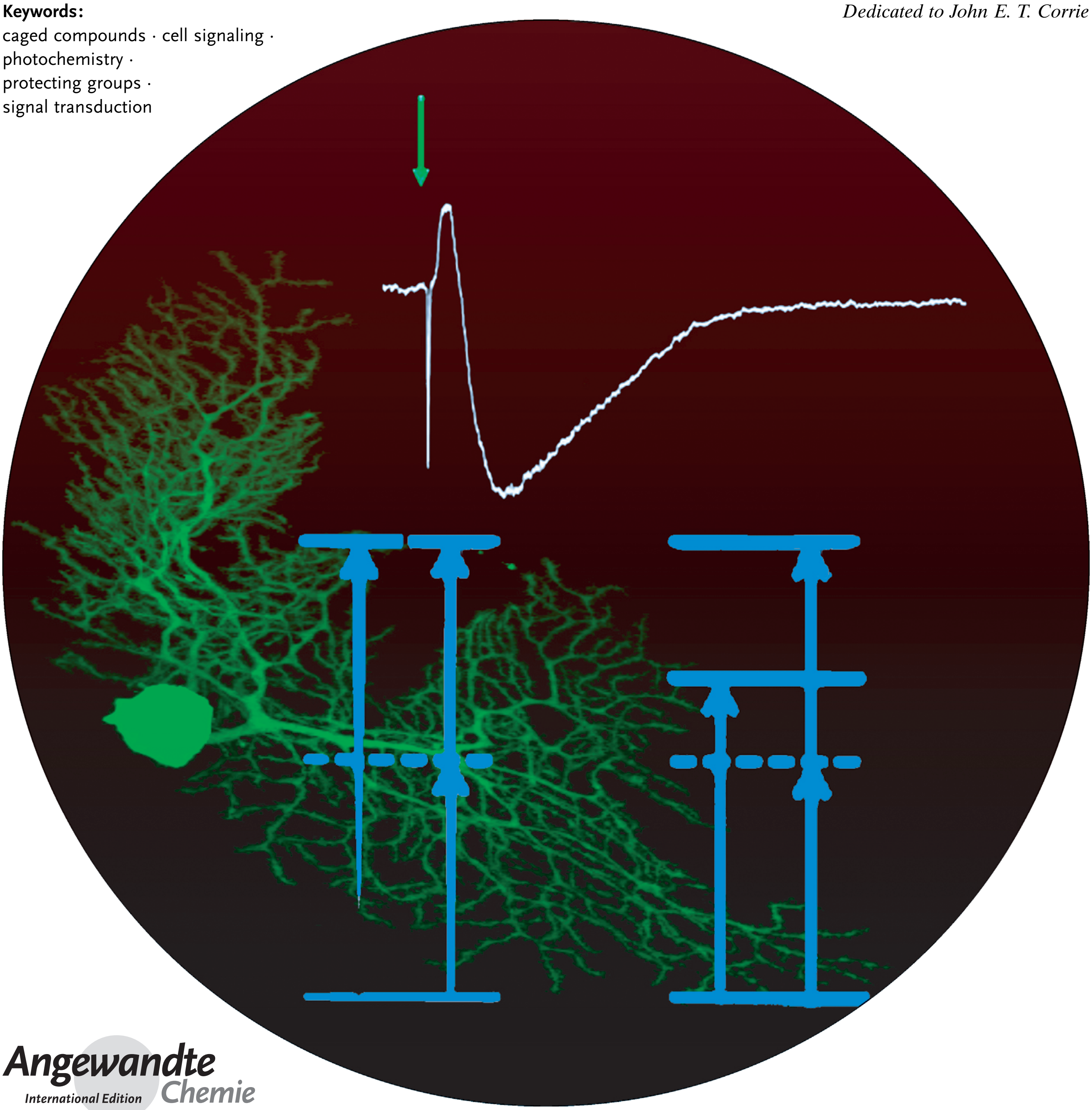
Keywords:
caged compounds · cell signaling · photochemistry · protecting groups · signal transduction

Dedicated to John E. T. Corrie



Molecular systems that can be remotely controlled by light are gaining increasing importance in cell biology, physiology, and neurosciences because of the spatial and temporal precision that is achievable with laser microscopy. Two-photon excitation has significant advantages deep in biological tissues, but raises problems in the design of "smart" probes compatible with cell physiology. This Review discusses the chemical challenges in generating suitable two-photon probes.

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1. Introduction

To understand the way in which receptors recognize and respond to different ligands, and to probe the signaling pathways they initiate, often requires experimental interventions with sub-micrometer and sub-millisecond resolution—this is the spatiotemporal scale of physiological processes such as synaptic transmission and intracellular second messenger signaling. The challenge for the experimenter is to perturb the biological system with a concentration jump of the physiological ligand, or a cofactor or antagonist, with timing and spatial dimensions that mimic the physiological process. This approach yields kinetic and mechanistic information, and also permits pharmacological or other interventions that would normally be impractical. In photochemistry, flash photolysis is used to initiate reactions to study their kinetics, and has been applied to ligand–receptor interactions in biology for 30 years, since the first publication of "caged" ATP by Kaplan et al. in 1978.[1] The idea is straightforward: the compartments containing the receptors are flooded with an inert but photolabile precursor of a ligand and the ligand is released with a spatially defined pulse of light once the "cage" is diffusionally equilibrated. In cell signaling, the localization, speed, and efficiency of physiological processes are achieved by reducing the spatial scales to sub-micrometer dimensions. Modern microscopy has developed to a level where commercial microscopes can accurately place precisely timed light spots within the tissue—a requirement for laser scanning confocal and two-photon microscopy. Consequently, photolysis ("uncaging") combined with two-photon microscopy can achieve high spatiotemporal resolution and is compatible with fluorescence microscopy. Caged compounds allow localized photorelease of ligands from biologically inert precursors;[2] actuators that convert light energy into motion and "photoswitches" introduced in light-gated receptors, channels, or enzymes permit alternate initiation and inhibition of the underlying biological process.[2j,3]

Uncaging has mostly been applied at the primary, usually near-UV, wavelengths with one-photon excitation (OPE), and is generally efficient enough for experimental application over the whole concentration range required. Problems of "inner filtering" by the cage solution, which is encountered at high concentrations and long path lengths, can simply be offset by using excitation wavelengths away from the maximum absorption and increasing the applied energy.[4] However, photolysis by scattered photons severely limits the spatial localization beyond a depth of 15–20 μm in biological tissues. The advantages of two-photon excitation (TPE) are twofold: 1) the reduced scattering of the near-IR photons in turbid biological tissues gives a two- to threefold increase in depth compared with near-UV excitation, and 2) two-photon excitation gives better definition of the focal spot because, unlike near-UV photons, the scattered IR photons do not excite.[5] The improvement anticipated is substantial, as seen in fluorescence imaging applications, where the depth of focal excitation is increased from 20 μm to several hundred micrometers by TPE.

With the advent of readily available fs-pulsed lasers, two-photon technology has the potential to revolutionize cell physiology and neurosciences. However, two-photon absorption and excitation is intrinsically much less efficient than its one-photon counterpart, especially with non-optimized reagents. Consequently the development of more-efficient two-photon probes for photolysis and photoswitching is needed to exploit the full potential of TP activation.[2g,i,6] The improvement of two-photon activation requires new approaches, mainly to greatly improve the two-photon absorption (TPA) similar to that of widely used fluorophores, while retaining the fast response rates, the high water solubility, the hydrolytic stability, and the biological inertness seen with many of the current near-UV probes. These approaches are reviewed here.

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2. Theoretical Basis of Two-Photon Absorption

Two-photon absorption is a nonlinear process in which two photons are absorbed simultaneously by the same molecule (Figure 1). It involves a "virtual state" with energy corresponding roughly to the half of the energy of the final allowed state, either the S_1 level for nonsymmetric compounds or S_2 level for centrosymmetric compounds. This virtual state is not related to any resonance of the chromophore and its lifetime is very short; consequently, TPA is very unlikely to occur ($\alpha_2 \ll \alpha_1$). In practice, this phenomenon is only commonly observed at the very high photon flux in the focus of a laser beam. The simultaneous nature of TPA implies that this process depends quadratically on the light intensity, and is related to the probability that two photons are localized in the same restricted space at the same time. Finally, TPA wavelengths are red-shifted, with the position of the maximum depending on the symmetry of the chromophore. One- and two-photon transitions are the same for dipolar noncentrosymmetric chromophores, which implies that the TPA maximum is twice that of the one-photon counterpart; the one and two photon transitions are distinct for symmetric quadrupolar chromophores.[7]

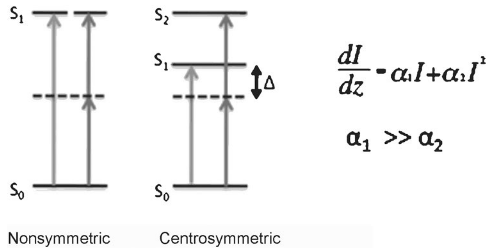

Figure 1. One-photon absorption (OPA; blue) and two-photon absorption (TPA; red) of dipolar (noncentrosymmetric) chromophores and quadrupolar (centrosymmetric) chromophores.

TPA is well-adapted to biological imaging and to the activation of molecular probes for a number of reasons. 1) The quadratic dependence on the light intensity restricts the excitation to a small volume at the focal point of the laser. This quadratic dependence also means that scattered photons alone do not excite away from the focus. 2) The red-shifted absorption maxima are in a wavelength range of 750–1100 nm; in this region, tissues are relatively transparent, because both tissue absorption and scattering are minimized. 3) TPA absorption is less efficient than one-photon absorption and, as few biological chromophores are strong two-photon absorbers, the use of TPA in combination with highly absorbing probes should reduce photodamage.

Structural factors that influence the TPA of a molecule have been studied extensively and are now reasonably well understood;[7,8] the TPA cross-section can now be predicted to a fairly high precision by computation.[9] The key parameters of increased TPA are extended conjugation (Figure 2) and the presence of strong donor and/or acceptor couples that

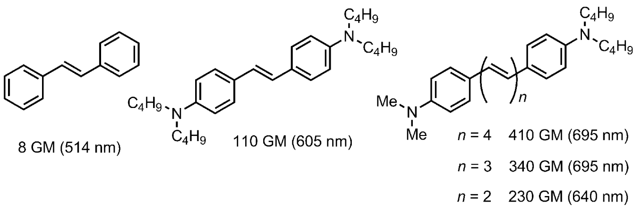

Figure 2. The effect of the donor and/or acceptor groups, and the effect of the conjugation length on the TPA absorption.[12]

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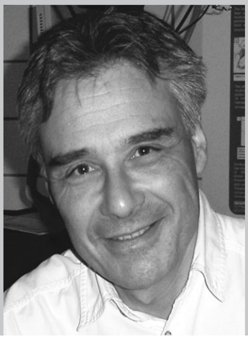

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promote strong internal charge transfer (ICT; Figure 2);[10,12] The strength of the polarization can also be evaluated by using Hammet parameters.[11]

Efficient conjugation requires planarity. This can be achieved by introducing rings or by complexation of a flexible chromophore to constrain a conformation (Figure 3).[13]

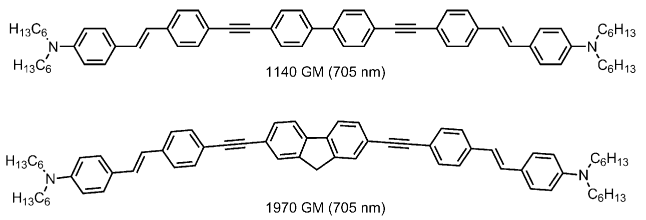


Figure 3. Improving the molecular planarity allows more efficient conjugation and π-electron delocalization, and thus TPA.

Further parameters are molecular symmetry and the presence of multibranched or oligomer structures, which may induce "cooperative effects"[7] and can induce TPA enhancement. For example, in quadrupolar chromophores of general structures D-π-D, D-π-A-π-D, A-π-A, or A-π-D-π-A (D: donor, A: acceptor) the presence of the excited state S_i, which corresponds to the first excited state populated by one photon, increases TPA through a "resonance enhancement" effect when the energy difference Δ between S_1 and S_2 becomes small (Figure 1). Likewise, "cooperative effects" are commonly observed between branches in multibranched chromophores.[8b] These systems are usually constituted from push-pull monomer chromophores assembled around a central acceptor or a donor core. Cooperative effects may arise from "through-bond" or "through-space" interactions; several explanations have been suggested to rationalize these phenomena, such as excitonic coupling,[14] vibronic coupling,[15] and extension of conjugation.[10] Classical multibranched structures are tripodes,[16] oligomers, and dendrimers.[17]

Molecules with high TPA cross-sections are usually large, highly conjugated hydrophobic compounds. In neuroscience, in particular, high lipophilicity is likely to have an effect on the electrical excitability and other aspects of synaptic communication. The correlation between lipid–water partition and anaesthetic-like activity among series of aliphatic or aromatic reagents is well-known. The requirement of a large π delocalization is difficult to combine with the high water solubility needed for biological application, and significant effort has to be undertaken to develop efficient water-soluble TPA chromophores with good properties in aqueous solution. Other approaches consist of using hydrophilic delivery agents such as nanoparticles, micelles, and biopolymers to deal with this problem.[18] However, in applications requiring free diffusion to facilitate high rates of photolysis, for example in synaptic transmission, or in applications subject to steric restraints, increasing the bulk of the probe may reduce the photolysis rates and hinder access to the compartments required.

The photochemical efficiency (δ) of a TPA process is determined by the two-photon absorption cross-section (σ_2), which relates the rate of absorption to the square of photon flux and has units of GM ($1\,\mathrm{GM} = 10^{-50}\,\mathrm{cm}^4\,\mathrm{s}^{-}\,\mathrm{photons}^{-1}$; named in honor of Maria Göppert-Mayer, who set the theoretical basis of the TPA process),[19] and is multiplied by the quantum yield (Φ), of the process; for example in uncaging reactions, Φ_υ is the fraction of excited chromophores that undergo photolysis and liberate free ligand. The photochemical efficiency as measured by the two-photon photolysis cross-section is thus given by $\delta = \sigma_2\,\Phi$. The photochemical efficiency can be measured directly—as the TP uncaging cross-section δ from the rate of ligand release, or cage loss determined by chemical analysis after a standardized exposure. Alternatively, indirect measurements can be made in two steps by measuring the TPA cross-section and the quantum yield independently. As a result of the difficulty of measuring quantum yields under two-photon conditions, the value derived from one-photon photolysis is used with the assumption that one- and two-photon absorption populate the same excited states and therefore induce the same uncaging quantum yield.

Highly localized and rapid uncaging often requires high average light intensities and a high concentration of the caged compound (in the mM range). These requirements present potential problems of phototoxicity and unwanted effects from the cage on both the target and other types of receptors. The minimum efficiency required is determined by the maximum light intensity tolerated by the system during an experiment. For OPE, this has been estimated as 5–10 $\mathrm{mW\,\mu m^{-2}}$ for short sub-ms pulses in intracellular photolysis experiments. The intensity required for full conversion of a commonly used cage, MNI-glutamate, by OPE has been estimated as 0.4 $\mathrm{mW\,\mu m^{-2}}$ at 360 nm and 2 $\mathrm{mW\,\mu m^{-2}}$ at 405 nm in 0.1 ms pulses.[4,20] These values are well within the toxicity limit. For TPE, the average power tolerated under photolysis or imaging conditions has also been estimated as about 5 mW (with standard Ti:S laser beam parameters and a high numerical aperture microscope objective producing a sub-micrometer focus). The studies also show a sharp threshold for toxicity and a multiphoton-dependence on intensity.[21] The multiphoton character of the phototoxicity suggests that longer laser pulses with reduced maximum intensity may be less damaging at comparable levels of induced fluorescence in imaging, an effect that may also apply to photolysis. In other types of experiment in developmental biology, in which the timescale of the experiments is much longer, phototoxicity has been reported at lower intensities, but here temporal resolution is less important than spatial.

Two-photon excitation is inherently inefficient and is observed at nontoxic intensities only in the focal volume of high numerical aperture (NA) microscope objectives. For submicrometer, femtoliter volumes, the diffusional exchange between the excitation volume and the surrounding solution has a characteristic time constant in the range of 100–400 μs, depending on the diffusion coefficient of the cage in the tissue. Diffusion determines the rate at which the photolyzed caged compound is replaced in the two-photon volume from outside, and also the rate at which activated intermediates and products are lost. Since the maximum photon flux that avoids phototoxicity is known, the minimum TP photolysis cross-sections required to achieve rates that produce 50% conversion of the cage concentration in the volume can be calculated. These were determined as 3–30 GM, depending on the diffusion coefficient of the cage. The localization of the photolysis reaction to the excitation volume requires that the rates of product release in the fragmentation reaction are fast compared with diffusion, thus requiring rates of 10000 s^{-1} or greater, and furthermore that exposure times longer than the diffusion time constant will lead to delocalization of the released ligand into adjacent regions.[21,22]

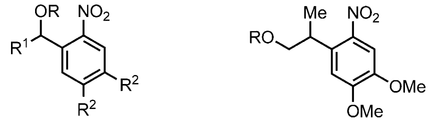
Although commonly available near-UV cages are efficient in OPE, their TPE cross-sections are generally less than 0.1 GM, and the dark fragmentation rates in many cases are too slow for localization to the excitation volume. These parameters are discussed in the next section in relation to currently available cages.

3. Two-Photon-Activatable Caged Compounds

Caged compounds currently in use for TP photolysis are derived from photochemical systems developed for near-UV-absorbing protecting groups. Although several photosensitive protecting groups have been adapted for cell physiology, none of them adequately satisfy the criteria needed for application in biological conditions and for two-photon photolysis. The properties of these caging groups are reviewed here.

3.1. Nitrobenzyl

Nitrobenzyl-derived cages (NB, **1a**; Figure 4) were first developed for release of biologically active ligands with near-UV photolysis in the 300–400 nm wavelength range.[23] Some of the currently used NB-derived protecting groups are depicted in Figure 4. They have been applied to the liberation of phosphates, carboxylic acids, amides, alcohols, and carbonyl functions, as well as of metal ions with the help of photolabile chelators; several derivatives are commercially available. However, these cages show poor performance under TPA conditions, with $\delta_u < 0.1$ GM at 710–740 nm. The efficiency of **1a** was improved by replacing the benzyl group by a homobenzyl function, such as in **1f**,[24] or by the addition of an electron-withdrawing group, such as a tribromo- or trifluoromethyl, in the benzylic position.[25] These modifications improved the photolysis efficiency by a factor of up to 3. The addition of methoxy groups such as in **1d–f** resulted in a red-shifted absorption while the quantum yield of the photolysis Φ_u was in general reduced by one order of magnitude compared to that of NB. The presence of the methoxy groups accelerated the dark fragmentation rate of the photolysis.[26]

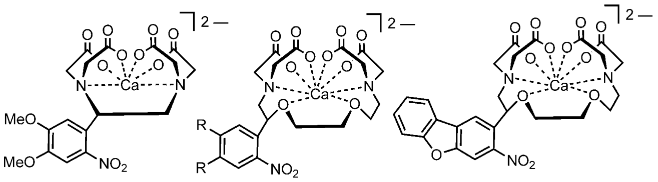


1a R^1 = H; R^2 = H (NB-OR)
1b R^1 = CO_2H; R^2 = H (CNB-OR)
1c R^1 = Me; R^2 = H (NPE-OR)
1d R^1 = H; R^2 = OMe (DMNB-OR)
1e R^1 = Me; R^2 = OMe (DMNPE-OR)
1f

Figure 4. Some of the currently used nitrobenzyl-derived protecting groups.

NB-derived caged neuroactive amino acids generally show poor hydrolytic stability. A carbamate linker between the alcohol and amino group generates stable, efficient caged amino acids for OPE, but their slow fragmentation kinetics at pH 7.4 of approximately 20 s^{-1} restricts their use to slow signaling processes.[23]

Nitrobenzyl-based chelators played a central role in the development of Ca^{2+}-selective cages (Figure 5).[27] The chelator is cleaved by photolysis to yield mainly iminodiacetic products with a much lower affinity for Ca^{2+} ions. Recently, however, a tricarboxylate with an intermediate affinity for Ca^{2+} ions was described, which was formed by a decarboxylation that accounted for about 17% of the photolysis of DM-nitrophen.[28] An advantage of the EGTA analogues such as in **2b,c** compared to the EDTA derivative **2a** is their lower affinity toward Mg^{2+} ions present under physiological conditions (Figure 5); however, the change in affinity for Ca^{2+} is less. These compounds show low TP uncaging cross-sections (in the range of 0.01 GM) and are generally used with near-UV excitation. The nitrodibenzofuran-derived EGTA **2d** (NDBF-EGTA)[29] is an exception, as this compound has δ_u = 0.6 GM at 710 nm. This NDBF chromophore has also been used to cage iron[30] and also for the caging of nucleotides[31] and IP_3.[32]



2a DM-nitrophen-Ca^{2+}
2b R = H; NP-EGTA-Ca^{2+}
2c R = OMe; DMNPE-4-Ca^{2+}
2d NDBF-EGTA-Ca^{2+}

Figure 5. Nitrobenzyl-derived Ca^{2+} chelators.

In some cases, unexpectedly high δ_u values have been reported for NB-caged chromophores. In NP-linked coumarins (**3**, **4**; Figure 6), a δ_u value of ≤ 0.68 GM was observed and attributed to photochemical cooperation between the cage and the leaving group. The effect was termed "substrate-assisted photolysis",[33] and attributed to the reverse energy transfer (i.e. from the lower energy to the higher one) from the coumarin to the NB group.

Similar cooperativity was observed in the photolysis of DMNB-caged *N*-vanillyl nonanoylamide (**5**, $\delta_u \leq 0.25$ GM, Figure 6)[33] and in retinoic acid (RA) coupled DMNB (**6**, Figure 6) used for the in vivo activation of the retina dorsal cells of zebrafish.[34] Likewise, NPE-caged hydroxypyrene

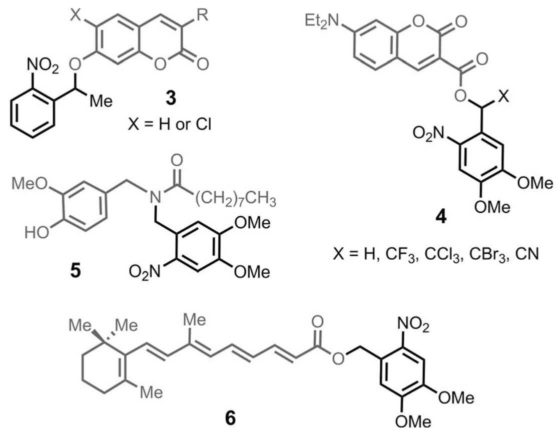


Figure 6. Substrate-assisted photolysis of nitrobenzyl-derived caged complexes.

trisulfonic acid (HPTS), a green fluorophore completely quenched by the NPE caging group shows a maximum photolysis efficiency at 405 nm, instead of in the near-UV region normally characteristic of the NP chromophore. Furthermore, the photolysis mechanism shows differences between the one-photon and two-photon excitation: faster fragmentation and pH independence is found with TP photolysis, in contrast to the strong pH dependence of the one-photon fragmentation.[21]

The influence of the conjugation length of the π system on TP absorption σ_2 has been studied.[25] Although NB derivatives such as **7** showed high absorption cross-sections ($\sigma_2 =$ 20–50 GM) at 730 nm and 800 nm, the uncaging cross-sections (δ_u) were comparable to that of the parent NB. This may be attributable to competing relaxations, such as fluorescence arising from the greater conjugation length. Better results were obtained with the 3-(2-propyl)-4′-methoxy-4-nitrobiphenyl (PMNB) derivative **8a**, and with the more water soluble tri(ethyleneglycol) derivative **8b** that showed a TP photolysis cross-section of 0.45 GM at 800 nm (Figure 7).[35]

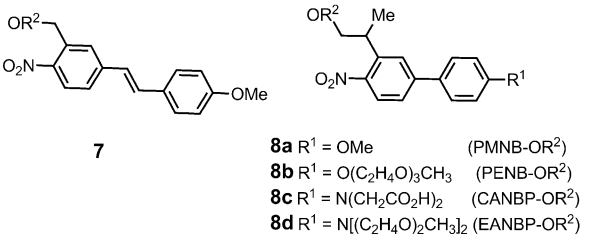


Figure 7. Nitrobenzyl-derived caged compounds having extended π systems.

Modification of the alkoxy donor group by a functionalized dialkylamino group, such as in **8c** (CANBP) or **8d** (EANBP), offered unprecedented (up to 11 GM) TP uncaging cross-sections at $\lambda = 800$ nm with improved aqueous solubility (up to 10 mM).[36] The TP excitation of CANBP-GABA and EANBP-GABA enabled rapid and spatially controlled release of GABA in intact brain slices.[36a]

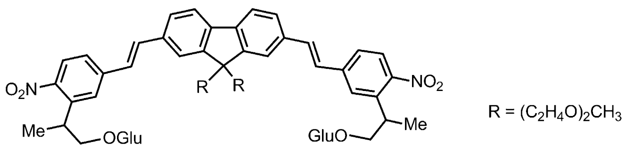
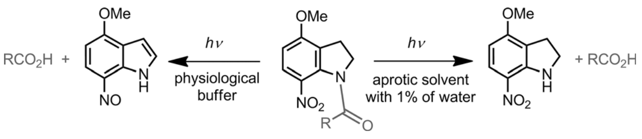


Figure 8. The symmetric quadrupolar NPE analogue BNSF-Glu$_2$.[35]

Another successful modification of nitrobenzyl-derived "cages" was realized by building a quadrupolar structure around a fluorene core (BNSF-Glu$_2$, Figure 8). The BNSF-caged glutamate displays a TP uncaging cross-section of 5 GM—one of the highest—at $\lambda = 800$ nm.[37] The planar fluorene core in the extended conjugation of this structure is probably the main contributor to the increased light-harvesting ability. The BNSF-Glu$_2$ does not allow for a quantitative release of the neurotransmitter (60 %) because of a competing photodegradation pathway, however, it is sparingly soluble in aqueous media (0.1 mM). Moreover, the slow dark fragmentation rate of 1000 s^{-1} that is characteristic of the 2-(o-nitrophenyl)propyl series prevents adequate localization to the TP excitation volume.

3.2. Nitroindoline

7-Nitroindoline (NI) derivatives were introduced by Amit et al. in the mid-1970s as carboxy protecting groups for use in aprotic solvents.[38] The photochemical properties were considerably improved by Corrie and co-workers to produce caged carboxylic acids for photolysis in aqueous solution and specifically adapted to the photorelease of neuroactive amino acids.[23,39] Importantly, the nitroindoline caged amino acids, unlike the nitrobenzyl and coumarin analogues, showed negligible hydrolysis at physiological pH. Furthermore, the fragmentation rate is high (5×10^6 s^{-1}, half-time 150 ns), which is fast compared to the activation kinetics of neuroactive amino acid receptors, and the quantum yield is 0.085, with release being stoichiometric with cage consumption. The effect of substitutions on the indole core was examined, and maximum photolysis efficiency was found with 4-methoxy-7-nitroindolinyl (MNI) derivatives.[39b] Mechanistic investigations have shown that the product of the photolysis of 7-nitroindolines depends on the medium and is different when the irradiation is performed in an aprotic solvent or in water. In an aprotic solvent with about 1 % water, 7-nitroindoline is formed through a solvent-assisted mechanism, whereas in water the photoproduct is the substituted 7-nitrosoindole (Scheme 1), formed without intervention of the solvent.[39a] Evidence based on the use of triplet sensitizers showed that photocleavage proceeds via a triplet state of the carboxylic nitronic anhydride intermediate. Subsequent release is by hydrolysis in organic media or by deprotonation at C(2) in

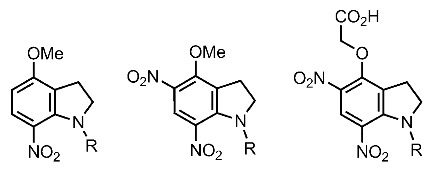


Scheme 1. Differences in the photolysis of the MNI-caged carboxylates in an aprotic solvent or in water.

aqueous solution, thereby resulting in the formation of the stable nitrosoindole end-product in physiological solution.

Modifications of the nitroindoline chromophore with substitutions that do not influence its photochemical properties have been applied systematically to minimize the pharmacological action of the NI-caged GABA and NI-glycine analogues on target receptors. This has resulted in the NI-caged inhibitory transmitters DPNI-GABA and DPNI-glycine with alkyl bisphosphate substutuents in the 4-position.[39c]

The 4-methoxy-7-nitroindolinyl glutamate (MNI-Glu, Figure 9) is widely used in neuroscience for OPE and TPE photolysis. Although the two-photon uncaging cross-section is small (0.06 GM at 720 nm), the nitroindoline-caged L-glutamates are sufficiently inert at glutamate receptors to be tolerated at high concentration, thus providing some compensation for the inefficiency. They have been used in many studies of glutamate signaling in neurons.[40]



9a MNI-R **9b** MDNI-R **9c** CDNI-R

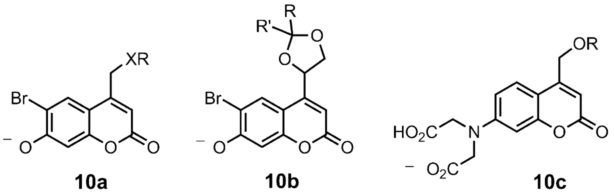
Figure 9. 7-Nitroindoline-derived caged compounds.

5,7-Dinitroindolinyl-L-glutamates, exemplified by 4-methoxy-5,7-dinitroindolinyl-glutamate (MDNI-Glu, **9b**) and 4-carboxymethyl-5,7-dinitroindolinylglutamate (CDNI-Glu, **9c**; Figure 9) were evaluated independently in two studies,[41] and found to be about twice as sensitive to near-UV excitation as MNI-glutamate. However, the stoichiometry of L-glutamate release was reported to be 0.7, and mixed nitro and nitroso photoproducts were obtained, thus differing from the near stoichiometry and sole nitroso product of mononitroindolines in aqueous solution.[41b] The enhanced uncaging was attributed to a greater quantum yield in one study,[41a] and to the larger absorption coefficient in the other.[41b] Although the photolytic efficiency of the dinitroindolines is 3.5-fold greater than the mononitro derivatives, the reduced stoichiometry presents an uncertainty in their use.[41b,42]

Although MNI-caged glutamate is the most widely used cage for TP photolysis, a major shortcoming of this system is the small two-photon cross-section, measured as around 0.06 GM for MNI-glutamate and estimated to be about threefold higher for the dinitro analogues. An interesting approach to improve the efficiency of nitroindoline photolysis is the use of an intramolecular triplet sensitizer, a benzophenone, linked to the nitroindoline.[43] Although this substantially increased the efficiency of one-photon photolysis, it shifted the peak OPA wavelength to 300 nm and the peak TPA to 580 nm, which is outside the range of Ti:S pulsed lasers. However, this is a promising strategy to improve the TPE of nitroindoline reagents.

3.3. Bromohydroxycoumarin[44]

Historically the first caged ligand with a TP uncaging cross-section of $\delta_u \geq 1$ GM was developed from 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) derivatives.[45] The progress of the photolysis can be easily followed by photometric monitoring, as the coumarin photoproduct is highly fluorescent. However, the ester derivatives proved hydrolytically unstable under physiological conditions. Although L-glutamate linked by the amine function, through a carbamate space, showed increased stability (Figure 10)[45] and had the same photolysis cross-section, it resulted in a slower glutamate release (a few ms at pH 7.2) and consequently reduced the localization of glutamate release. Di- and trihalogenated coumarin derivatives resulted in better TP uncaging cross-sections, but with less clean fragmentation reactions. According to the suggested mechanism, the bromine substituent may lower the pK_a value of the phenol, thereby promoting the formation of the more strongly absorbing anion and also decreasing the lipophilicity of the compound.[46] Recently, 7-aminocoumarin-based phototriggers have attracted much attention because of their higher photolysis efficiencies, long-wavelength absorption, and efficient fluorescence visualization.[34,47]



10a Bhc-XR **10b** **10c** BCMACM-OR

XR = *O*-acyl, *O*-carbamoyl, *S*-alkyl

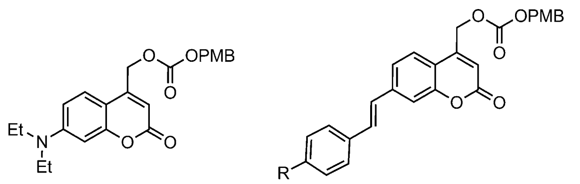
Figure 10. Caged compounds derived from bromohydroxycoumarin (Bhc) and aminocoumarin (AC).

The flexibility of coumarin derivatives was validated[48] by the liberation of alcohols,[48a] diols,[48b] and thiols.[48c] Three-dimensional chemical patterning of amines within an agarose hydrogel was realized by photolysis of the linked Bhc by two-photon excitation.[48d] Aldehydes and ketones could also be released from the corresponding acetals and ketals.[48e] Also, a photoacid (i.e. a compound that becomes strongly acid on irradiation) was developed from amino-substituted coumarin, and was probed under TPA conditions, with the proton release monitored by the pH-sensitive fluorescein isothiocyanate dextran.[48f]

Bhc cages have been used as cell-membrane-permeant agents in the intracellular TP photolysis of cyclic nucleotide secondary messengers;[49] for the liberation of GABA in whole-cell recorded hippocampal CA1 pyramidal cells;[50] for the inhibition of protein synthesis by the in vivo release of anisomycin;[51] for the photolysis of Bhc-derived progesterone regioisomers;[52] and for the in vivo activation of a competitive inhibitor of the inducible nitric oxide synthase (iNOS).[53] The more water-soluble **10c** (BCMACM) was used for the liberation of a cGMP analogue, thereby triggering a Ca^{2+} ion influx into CNG receptor transfected HEK293 cells.[54]

Bhc cages have high TP uncaging cross-sections of $\delta_u \leq 2.28$ GM, and have fast photolysis kinetics. The coumarin photoproduct is highly fluorescent, an advantage for following the photolysis reaction, but limits the use of these cages in conjunction with fluorescent indicators. However, photolysis of Bhc-caged compounds may give rise to the formation of considerable amounts of by-products, depending on the substrate and the conditions.[48c] Moreover, ester derivatives are usually sensitive to hydrolysis, thus compromising their use under physiological conditions.

Conjugated coumarin-based TP phototriggers with *p*-substituted styryl groups at the C(7)-position have been prepared to increase the TP sensitivity; these compounds have longer absorption wavelengths and larger δ_u values than nitrobenzyl-derived cages.[55] While high TPA (σ_2) cross-sections were obtained at 800 nm, the *p*-ethoxy derivative **12a** did not undergo fragmentation, and the efficiency (δ_u) of the *p*-dimethylamino derivatives **12b** was twice as high (up to 0.26 GM versus 0.12 GM) as the parent coumarin derivative **11**, without the styryl appendage (Figure 11).



11 δ_u = 0.12 GM (800 nm)

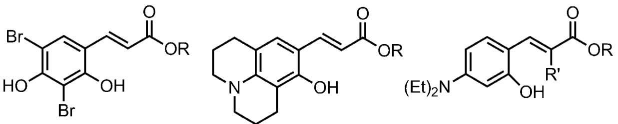
12a R = OEt; δ_u = 0 GM (800 nm)
12b R = NMe$_2$; δ_u = 0.26 GM (800 nm)

Figure 11. Comparison of coumarin-based TP phototriggers with *p*-styryl substituents. PMB = *p*-methoxybenzyl.

A TP-initiated self-immolative approach was also developed by using a Bhc-derived dendrimer structure that translates a single triggering event into a cascade of molecular rearrangements.[17b] The method allowed the release of multiple terminal groups. It is important to note, however, that the overall cascade degradation process is relatively slow, as the rearrangement occurs in series. Nevertheless, this is a useful strategy for increasing the overall biological response to light and achieving sustained release.

3.4. o-Hydroxycinnamate

o-Hydroxycinnamate (OHC) derivatives were introduced by Porter and co-workers in 1988,[56] and were developed further for the photorelease of alcohols.[57] These show particularly high uncaging cross-sections ($\delta_u \leq 4.7$ GM at 750 nm in acetonitrile, Figure 12). The more water soluble 3,5-dibromo-2,4-dihydroxycinnamate cage **13a** was used for physiological experiments ($\delta_u = 1.6$ GM at 750 nm) and validated in an embryo zebrafish model (60 min irradiation at 750 nm, using < 5 mW maximum power in 10 μM aqueous solution).[57b] Notably, many *o*-hydroxycinnamate derivatives have high cell membrane permeability, which permits utilization of the spatial localization of TPE to restrict activation to single cells.



13a **13b** **13c** R' = Me, NHAc or H

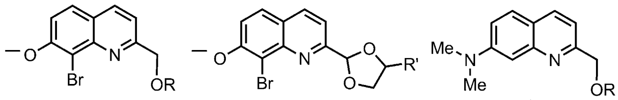
Figure 12. Photoactivable *o*-hydroxycinnamate derivatives.

o-Hydroxycinnamates such as **13a–c** (Figure 12) undergo *E* to *Z* photoisomerization by irradiation followed by thermal lactonization, thereby releasing the substrate (ROH) and forming a coumarin. Kinetic analysis under OPA conditions showed that the rate of the *E*/*Z* photoisomerization and the thermal transformation of the *ortho*-hemiester to the coumarin are the slow steps of the photorelease.[57a] Substitution of the cinnamate double bond (R^1 = Me or NHCOMe) accelerates the fragmentation reaction by approximately one order of magnitude. The rate of the thermal process under TPA conditions is considerably higher than the photoisomerization step. The substrate release under in vivo conditions was slow, however, and was in the range of 10^{-2}–10^{-3} s^{-1} at 750 nm at a maximum power of 4.5 mW.

The high cross-section and the ready availability of the OHC platform from simple synthons are notable advantages;[58] however, their use is limited to applications where slow substrate release is not detrimental.

3.5. Bromohydroxyquinoline

8-Bromo-7-hydroxyquinoline (BHQ) was introduced by Fedoryak and Dore.[59,60] BHQ-OAc (**14a**, Figure 13) has a δ_u value of 0.59 GM at 740 nm in buffered aqueous solution, which is comparable to that of Bhc-OAc ($\delta_u = 0.72$ GM) under similar photolysis conditions. However BHQ-OAc has the advantage of greater water solubility than Bhc-OAc and is less fluorescent, thus permitting its use in conjunction with fluorescent indicators. The photolysis of carboxylates (δ_u = 0.64–0.76 GM), phosphates (δ_u = 0.43 GM), and diols (**6b**, δ_u = 0.78–0.90 GM) have been reported.[46a] BHQ-OAc was photolyzed more rapidly than Bhc-OAc under UV irradiation, and was hydrolytically stable enough for use in physiological experiments.



14a
BHQ-OR

14b

14c
DMAQ-OR

R = phosphate, carboxylate or carbamate.

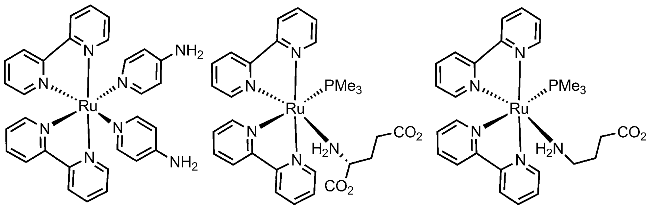
Figure 13. Bromohydroxyquinoline (BHQ).

The replacement of the C(8) bromine substituent by nitro, cyano, or chloro groups, or the replacement of the phenol hydroxy group by dimethylamino (**14c**) or sulfhydryl groups, resulted in diminished one- and two-photon efficiency compared to the parent BHQ-OAc.[61]

The photolysis of BHQ cages is believed to proceed via a singlet excited state through a solvent-assisted photoheterolysis (S_N1) and shows fragmentation on the sub-microsecond time scale.[46a] Consequently, replacement of the bromine by other electron-withdrawing groups that do not facilitate ISC but lower the pK_a value of the phenolic proton may be a viable strategy for preparing analogues.

3.6. Ruthenium–Bipyridyl Complexes

The initial photoexcited state in ruthenium–bipyridyl (RuBiPy) complexes (Figure 14) quickly evolves into a dissociative state, thereby affording clean and fast photorelease.[62] Ruthenium–bipyridyl complexes have been used to cage amine groups in different molecules.[63] Such complexes were used for the release of the potassium channel blocker 4-aminopyridine from **17a**.[64]



17a RuBi-4AP

17b RuBi-Glu

17c RuBi-GABA

Figure 14. RuBiPy-caged ligands.

A structurally similar RuBi-glutamate[65] **17b** and the caged GABA RuBi-GABA, (**17c**)[66] were used for the optical mapping of glutamergic and GABAergic receptors under one- or two-photon excitation. The TP photolysis cross-sections were measured as 0.1 GM by comparison with that of MNI-glutamate. As with MNI-glutamate, antagonism at GABA receptors and the low cross-section limits their use in TP photostimulation. Neuronal studies report high intensities and long exposures required to elicit spiking in the target neurons.[65]

3.7. Miscellaneous

Several TP-sensitive caged compounds such as the azide-derived calcium cage[67] and a TP-sensitive 2,6-dimethylnitrobenzene-based NO donor (Flu-DNB) were also developed.[68] A variety of NO-releasing cage compounds were also prepared from $Fe_2S_2(NO)_4$ clusters, known as the Roussin's red salt.[69] The general scope of these compounds remains to be addressed, however.

4. Specific Applications of Caged Compounds

4.1. Dynamic Studies for Following Diffusion and Trafficking

The photoactivation of caged fluorophores and photoactivatable genetically encoded fluorescent proteins[70] can be used to follow the diffusion and trafficking of tagged molecules.[71] This technique offers two advantages over the more conventional "fluorescence recovery after photobleaching" (FRAP) techniques. Firstly, the measurements have intrinsically a higher signal-to-noise ratio, as most photoactivatable fluorescent proteins produce little fluorescence before photoactivation. Second, photoactivation may produce fewer free radicals than photobleaching, thus inducing less phototoxicity. This approach has been used to measure spine/dendrite diffusional coupling with PA-GFP and with NPE-HPTS by using two-photon excitation for fast release of the small fluorophore HPTS (pyranine).

4.2. Syncronous Activation of Multiple Sites: Patterned Activation

The ability to achieve the patterned, almost synchronous activation of multiple sites, such as dendritic spines, permits studies on the temporal and spatial integration of inputs. This can be done either with fast scanning in two spatial dimensions or with holographic excitation in three dimensions. Patterned uncaging of caged glutamate in combination with electrophysiology has been performed in the preparation of brain slices either by using one-[72] or two-photon excitation,[73,74] and by using a spatial light modulator (SLM) based scanless TP microscope.[73] The scanning methods with two-photon excitation have successfully shown the way in which spatiotemporal integration can generate nonlinear responses to different input patterns.[40a,b] However, the main impediments to this approach are the high cage concentrations and long exposures required to avoid the phototoxicity resulting from the poor TP photolysis cross-sections of the available cages, such as MNI-glutamate. Improvement is required in the TP uncaging cross-sections to bring the exposures and concentrations down to acceptable, nontoxic levels while maintaining localization.[75]

4.3. Multimodal Photoregulation

The activity of two or more different molecular species could be photoregulated independently through the wavelength-selective response of different chromophores.[76] Selective activation may be achieved with reagents that are distinguished by pronounced differences in their photolytic quantum yields, in their sensitivity to two-photon (versus one photon) photolysis, or on the basis of their wavelength sensitivity. While multimodal photolysis by controlling the wavelength alone was achieved by OPA in the UV/Vis region,[76b,77] selective TPA is more difficult because TP absorption spectra are often broader and overlap. The two-color, two-photon uncaging of glutamate and GABA on the same neuron was achieved with single synapse precision by the selective activation of CDNI-caged glutamate and an aminocoumarin-caged γ-aminobutyric acid. The selectivity was achieved by combining the light wavelength with other experimental parameters such as the laser power and caged compound concentrations,[76a] as the CDNI-Glu was uncaged 38 times more effectively at 720 nm than at 830 nm, while *N*-DCAC-GABA was more efficient at 830 nm and used at twice the concentration. Thus, although the independent release of L-glutamate or GABA could be achieved, the experimental conditions were critical and the localization was compromised by the slow-releasing caged GABA used.

5. Reversible TP Photoswitches

Recent advances in fluorescence photoswitching are driven by the expansion of super-resolution microscopy,[78a] by the development of optical lock-in detection (OLID) imaging,[78] which allows the image contrast to be improved instead of the spatial resolution by modulating the fluorescence emission, and also by the increasing importance of engineered light-gated proteins in molecular biology.[2j] A general strategy for the preparation of engineered photoswitches is to noncovalently (intercalating) or covalently couple a photoisomerizable molecule (a "photoswitch") onto a biomolecule such as DNA, an enzyme, an ion channel, or a receptor to make it sensitive to light. In principle, the photoswitch can be attached in such a way that photoisomerization energetically favors one conformation over another, thereby producing a light-gated conformational change and switching between active/inactive or open/closed forms.[79] Alternatively, the photoisomerization may deliver or remove a ligand from a binding site on the channel or receptor, thereby regulating its activity.[2j] Although several chemical photoswitches have been tested, azobenzene and its derivatives appear best suited for biological applications. Although the TPE of azobenzene derivatives has not been reported,[79a] spyropyranes were examined recently. Spiropyrans (SPs) are molecular photoswitches that have been widely utilized to engineer photochromic materials, including organic polymers and organic–inorganic hybrid thin films. The alternating spyropyran (SP) and merocyanine (MC) sytems have been excited by 780 nm NIR two-photon and 488 nm single-photon excitation to induce reversible "on-off" fluorescence imaging of photoswitchable immunotargeted nanoparticles.[80]

6. Summary

The two-photon activation of caged ligands, actuators, and photoswitches combined with microscopy is potentially a powerful experimental tool to investigate signaling mechanisms with high spatiotemporal precision. It has the advantage over one-photon excitation of retaining high precision deeper within live tissues such as the brain. Furthermore, the photonics required have been mainly developed in two-photon fluorescence microscopes for imaging deep in live tissues. Although steady progress has been made in extending the range of one-photon photolysis, the design and implementation of two-photon probes that have high photolysis cross-sections combined with good biological properties has remained elusive. In contrast to TP fluorophores, the basic rules permitting the rational design of TP-caged compounds are less-well developed. Although several photosensitive protecting groups have been introduced, none of them adequately satisfies the criteria needed for both application in biological conditions and efficient two-photon activation. Phototoxicity limits two-photon irradiation with ultrafast-pulsed lasers to about 5 mW average power.[21] From the beam parameters of commonly used Ti:S mode-locked lasers and the constraints of reaction with diffusion in localization to the TP excitation volume, one can predict that two-photon photolysis cross-sections in the range of several GM are needed together with fragmentation rates of $>10000\,s^{-1}$. Cages currently available have cross-sections much less than this, in the range 0.01–0.1 GM at 720 nm, and fragmentation rates are typically less than $10000\,s^{-1}$. Increasing the cross-sections will likely increase the size and lipophilicity of the cages, thus requiring strategies to increase the aqueous solubility, as well as retaining high fragmentation rates. Next-generation TP-activatable probes that satisfy these requirements at wavelengths that are accessible with commercially available lasers would give an impetus to spatially resolved sub-micrometer photochemistry to learn about fast, localized processes in biological networks.

We acknowledge the generous financial support of the EU Strep "Photolysis" (LSHM-CT-2007-037765) grant, ANR (ANR-2010-BLAN-1436-01), and the CNRS.

Received: May 30, 2012
Revised: September 7, 2012

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