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Small photoactivatable molecules for controlled fluorescence activation in living cells

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ABSTRACT

The search for chemical probes which allow a controlled fluorescence activation in living cells represent a major challenge in chemical biology. To be useful, such probes have to be specifically targeted to cellular proteins allowing thereof the analysis of dynamic aspects of this protein in its cellular environment. The present paper describes different methods which have been developed to control cellular fluorescence activation emphasizing the photochemical activation methods known to be orthogonal to most cellular components and, in addition, allowing a spatio-temporal controlled triggering of the fluorescent signal.

1. Introduction

Green fluorescent proteins and their genetically modified constructs have revolutionized cell biology allowing to visualize and track down cellular proteins in their intact environment. The use of GFPs as a noninvasive tool for studying protein dynamics prompted the engineering of mutants with improved fluorescence properties (emission wavelengths, brightness and photostability).[1] During these investigations, the development of photoactivatable fluorescent proteins (PA-FPs)[2] represented a real break through in the field, allowing in particular to analyse dynamic cellular processes using sophisticated optical imaging techniques. A large series of genetic variants have been produced allowing to generate different classes of PA-FPs either switching on fluorescence, changing colour or leading to a reversible on/off system after photoactivation.[3] Despite these overwhelming successes, one might still ask the question how do such constructs alter the cellular responses of the GFP-appended proteins and in particular how affected are the dynamics of these cellular proteins, knowing the ~25 kDa molecular weight of the GFPs?[4] Clearly, knowing that only small molecule-based probes can be used in native tissue, there is a increasing need for small photoactivatable fluorescent molecules, provided these are cell permeable and can be attached covalently or reversibly, but specifically, to the target cellular protein. This article summarizes the different approaches for cellular fluorescence photoactivation which have been developed recently as well as the different small tags which have been designed for a selective protein recognition.

2. Results and discussion

2.1. Small fluorescent molecules for cellular imaging

The need for convenient and reliable tests of cellular actors stimulated the development of fluorescence-based technologies. These tests include fluorogenic enzyme substrates as well as fluorescent ion indicators[5–7] to generate, in a quantitative manner, a fluorescent signal either after enzymatic reaction or after specific ion recognition. Although, these fluorescent signals are used to monitor the activity of a cellular protein, these reporters have been covered by others and would exceed the scope of this review, therefore we will not further develop this aspect of fluorescence activation.

Most of these tests use small fluorescent molecules whilst the GFP's, taking advantage of the existence of a large colour palette, have been mainly used in FRET- and translocation-based systems to visualize dynamic cellular processes. Clearly, the main advantage of GFP's remain their convenient expression as fusion protein in most cellular systems, but despite the improvements displayed by recent genetic variants of GFP's,[1b] the need for small fluorescent molecules with optimized photophysical properties remains critical for many cellular studies.[8] The requirements for these fluorescent probes are multiple, their size should be small to minimize steric perturbations as well as to enable a better cellular penetration. The selected chromophores should be red-shifted emitting fluorophores with a high brightness which is defined as the product of the fluorescence quantum yield by the extinction coefficient at the excitation wavelength ($\Phi_{fl} \cdot \varepsilon_{exc}$, $M^{-1}\,cm^{-1}$). Another desirable property is a good photostability, knowing in particular that

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the GFP's are often prone to bleaching after sustained photoactivation. Finally, a large Stokes shift, the difference in nm between the absorption and emission wavelength maxima, will facilitate the fluorescent experimentations, that is, by preventing self-quenching phenomena. The largest caveats for these small molecules are their poor aqueous solubility, knowing that a high absorptivity does correlate with an increased conjugation of the chromophore involving often aromatic systems and decreasing thereof considerably their solubility in aqueous medium. The substitution of the chromophores with ionized functions, that is, carboxylates for fluorescein or rhodamine derivatives; sulfonates for Cy3 or Cy5 derivatives, does improve the solubility properties but it might also affect their ability to cross the cellular membranes by passive diffusion. The recent review by Lavis and Raines[8] summarized exhaustively and accurately the overall properties of small fluorescent molecules.

2.2. Cellular fluorescence activation—concepts

Many strategies have been developed to activate fluorescence in response to an external stimuli (pH, local environment, etc.), we will focus on activation processes using a photochemical reaction.

2.2.1. The uncaging concept

Uncaging refers to a process where a biomolecule, after having been rendered functionally inactive by chemical appending of a photoactivatable group, can be unleashed through a fast and efficient photolytic reaction. The chemistry involved in these derivatives and the accompanying photolysis reactions have been described in details in the literature,[9a–d] as well as the biological applications which have been recently reviewed.[9e,9f]

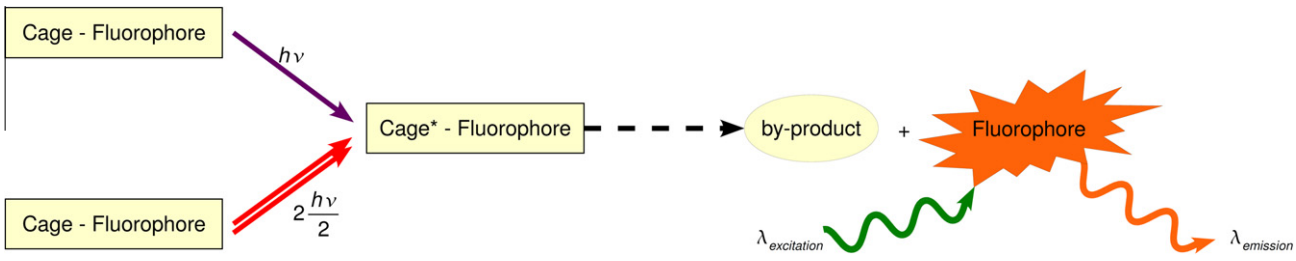
2.2.1.1. Fluorescence uncaging. Amongst the described applications of the uncaging concept, a spatio-temporal controlled release of fluorescence by photochemical activation does represent a powerful tool for cellular biologists, especially when the fluorescent signal allows the tracking of a cellular component in living systems.[9g] The experimental procedures have to be compatible with a biological environment, that is, the experiments require to be performed in a buffered aqueous medium and the excitation wavelengths should be above 350 nm to minimize damage to intracellular components (Scheme 1).

One-photon fluorescence uncaging. The light-induced fluorescence enhancement after an uncaging process is usually achieved by modifying a chemical function directly responsible for the fluorescent signal, that is, the aromatic hydroxyl or amino groups of usual fluorophores like fluorescein,[10] rhodamine,[11] Q-rhodamine,[12] resorufin[12] and hydroxycoumarin derivatives.[13] Most of these fluorescent derivatives used *o*-nitrobenzyl (*o*-NB) derivatives as photoremovable protecting groups. *o*-NB caging groups have also been used on TokyoGreen derivatives, fluorescein compounds with improved fluorescent properties.[14] Hydroxycoumarins were also modified with different photoremovable groups such *o*-nitrophenethyl derivatives,[15] a caging group which displayed remarkable photolytic properties for the release of the neurotransmitter glutamate.[16] Alternatively, the photochemical conversion of conjugated aromatic azido derivatives into corresponding anilino derivatives to generate fluorescent push-pull systems with high absorbance have been described recently.[17] Photochromic rhodamine derivatives have been developed recently with light-induced fluorescence activation[18] whilst a new kind of caged rhodamine derivatives were described using a diazoketone caging group incorporated into a spiro-9H-xantene fragment.[19] These rhodamine-NN derivatives are nonfluorescent at the excitation of their fluorescent analogues and they can be uncaged under convenient irradiation conditions. In comparison to the *o*-NB caging groups, the diazoketone is less sterically demanding, less lipophilic and may represent a way to circumvent the toxic nitroso by-products which are generated during the photo-cleavage of *o*-NB type caging groups. The diazoketone caging group may be exportable to other fluorophore scaffolds.

Figure 1 below summarizes the structures of the caged fluorophores which have been described in the literature.

Uncaging with fluorescent reporters. A series of articles have described the uncaging of chemical groups with concomitant formation of a fluorescent probe used as a reporter (Fig. 2). These molecules include the photochemical formation a diazabicyclo[2,2,2] derivative generated from a thiadiazolidinedione precursor,[20] a thioxanthone derivative from masked aromatic ketones,[21] coumarin derivatives generated from hydroxycinnamic esters used to cage alcohols[22] and xanthone derivatives used as a photolabile protecting group of carboxylic acids.[23] The fluorescent reporter approach is particularly attractive for an easy quantification of the photolytic reaction which is potentially very useful for biological applications. The major limitation concerning these probes is their excitation wavelength which are not well-adapted for in vivo experiments unless two-photon uncaging procedures can be applied.[22,24]

Two-photon fluorescence uncaging. After two-photon excitation, at wavelengths = $h\nu/2$, a molecule reaches an excited state similar to that of one photon excitation and can be subject to chemical reactions leading to bond cleavage. This is of particular interest in the rapid photo-liberation of biologically active substances (two-photon uncaging) and has been mainly applied for a spatio-temporal controlled release of neurotransmitters.[25] The main advantage of two-photon excitation is that it produces excited states identical to standard UV excitation whilst overcoming major limitations when dealing with biological materials, like spatial resolution, tissue penetration and toxicity. Due to moderate uncaging cross-sections ($\delta a \cdot \Phi u$ where δa is the absorption cross-section and Φu the uncaging quantum yield) described for the usual photoremovable groups,[25b,26] two-photon fluorescence uncaging, has been sparingly described in the literature by contrast to two-photon fluorescence excitation which has been widely developed for improved fluorescence imaging techniques[27] and displaying high



Scheme 1. Principle of fluorescence uncaging: one- or two-photon excitation of the caging group is followed by chemical cleavage leading to the release of the fluorophore together with a by-product.

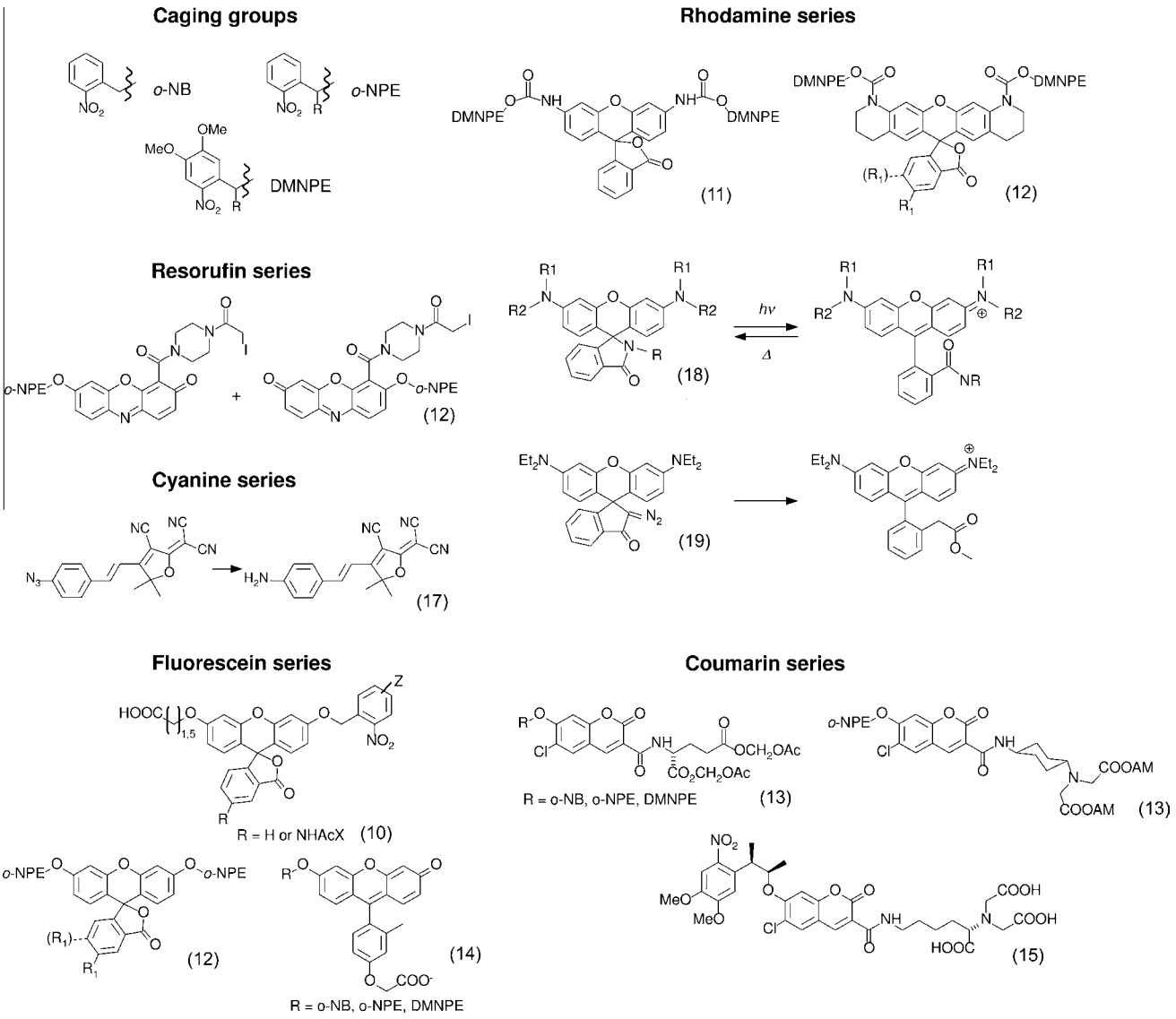


Figure 1. Structure of caging groups and caged fluorophores. Corresponding references are shown in brackets.

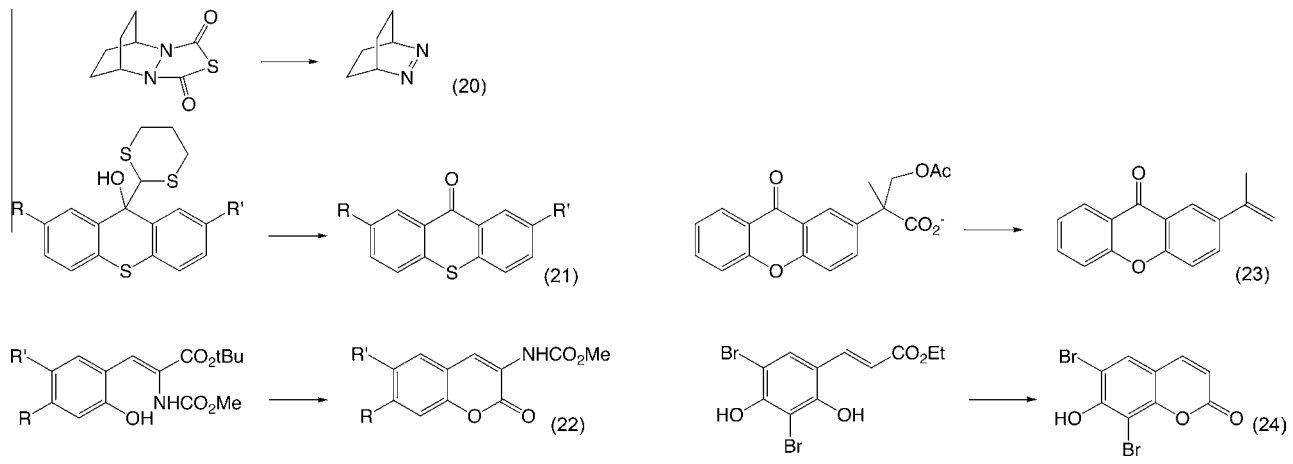


Figure 2. Structure of photoactivatable fluorescent reporters. Corresponding references are shown in brackets.

two-photon excitation cross-sections.[28] A main difference between two-photon fluorescence uncaging and two-photon fluorescence excitation is that, in the former case, the excitation process will be independently triggered by standard excitation allowing

subsequent biophysical experiments (single molecule fluorescence tracking, bleaching, etc.).

The few examples (Fig. 3) for two-photon fluorescence uncaging described in the literature include the *o*-NB[13] and the *o*-nitrophenethyl[15] hydroxycoumarin derivatives, whilst coumarins were also used as fluorescent reporters during two-photon alcohol uncaging.[22,24] To improve these photolytic properties, our laboratory synthesized a donor–acceptor biphenyl platform derived from the photoremovable *o*-nitrophenethyl group, which increased substantially the two-photon uncaging cross-sections, reaching a value of 3.4 GM at 740 nm.[29,30] Using this new caging group on a red-emitting acridinone fluorophore, we were able to unleash efficiently a fluorescent signal in HeLa cells by two-photon uncaging.[31]

2.2.2. Annihilation of quenching systems

2.2.2.1. Concepts. Besides fluorescence caging, which is a fluorescence extinction by direct chemical modification of the fluorophore, fluorescence extinction can be achieved by chemically linking a fluorophore to a quenching system via a linker. If the quenching system of such continuously quenched fluorophore can be effectively and specifically switched off (which then restores the fluorescence) as response to light, then the fluorophore-quencher pair is an interesting photoactivatable fluorescence reporter. The quenching mechanisms used in such fluorescence reporting systems are based either on a photoinduced electron transfer or on a resonant energy transfer.

2.2.2.2. Photoinduced electron transfer. During a photoinduced electron transfer an electron is transferred, after absorption of a photon of suitable energy, between the fluorophore and a partner of suitable orbitalar energy levels. The fluorophore and the partner for photoinduced electron transfer may be covalently linked but have to be disconnected from conjugation. The direction of the electron exchange depends on the reduction potentials of the implied species.

If it is the fluorophore that accepts an electron from a partner entity, the lowest singly occupied molecular orbital of the excited state of the fluorophore becomes fully occupied, and a fluorescent return to the ground state is no longer possible. The acceptance of an electron by the excited fluorophore results thus in a fluorescence quenching.

If the excited fluorophore donates its highest energy level electron to the partner entity, it thus looses the excitation energy. In this case too, the fluorescence is quenched by the photoinduced electron transfer.

A detailed description of the physical chemistry of the photoinduced electron transfer would however go beyond the scope of this article. If interested the reader may refer to the very informative literature existing on the subject.[32–36]

Most fluorescent sensor systems, which make use of a photoinduced electron transfer mechanism, have in common that the fluorescence is restored after a specific interaction of the quencher and an analyte. The reason for this is that the interaction with the analyte changes the redox potential of the quencher so that the photoinduced electron transfer is no longer possible and the fluorescence is restored. The two most prominent ways of annihilating a photoinduced electron transfer quenching system are either a chemical reaction (e.g., a Michael Type addition of a thiol on a maleimide analogue)[36–38] or the chelation of a cation (H^+, Na^+, K^+, Ca^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, etc.).[5–7] Since these fluorescence activations are not photoinduced, we will not discuss them further. Yet, a system kept out our attention where a classical fluorescein-analogue caging system is significantly optimized by finely tuning an intramolecular photoinduced electron transfer.[14]

In this study, three mono-caged fluorescein analogues (amongst these the caged 2-Me-4-OCH_2COOH TokyoGreen) have been developed, which had remarkably low fluorescence quantum yields before photoactivation, compared to a mono *o*-NB caged fluorescein (Fig. 4). This is of particular interest because mono *o*-NB caged fluorescein still has a residual fluorescence quantum yield of 0.117 before photoactivation, which makes it unsuitable for bioimaging, despite its satisfying uncaging rate. In the past, totally nonfluorescent double-caged fluoresceins have been used for cellular investigation.[11] A major drawback of these probes is that two caging groups have to be deprotected in order to regenerate the fluorescence. This entails a higher photo toxicity for the observed cells compared to a singly caged fluorophore. The mono-caged fluorescein ether described by Krafft et al., which was fixed in the lactone form, yields a fluorophore after photoactivation which is less fluorescent than fluorescein.[10]

Kobayashi and co-workers lowered the fluorescence of the fluorophore before photoactivation by using 2-Me-4-OCH_2COOH TokyoGreen instead of fluorescein. In the described compounds, the oxidation potential of the benzene was finely tuned in order to show a drastic increase in fluorescence after cleavage of the caging group.

Before photoactivation the singlet-excited state of the TokyoGreen fluorophore is quenched by an intramolecular photoinduced electron transfer from the phenyl group of the TokyoGreen to its xanthene moiety, and after photoactivation, the electron transfer

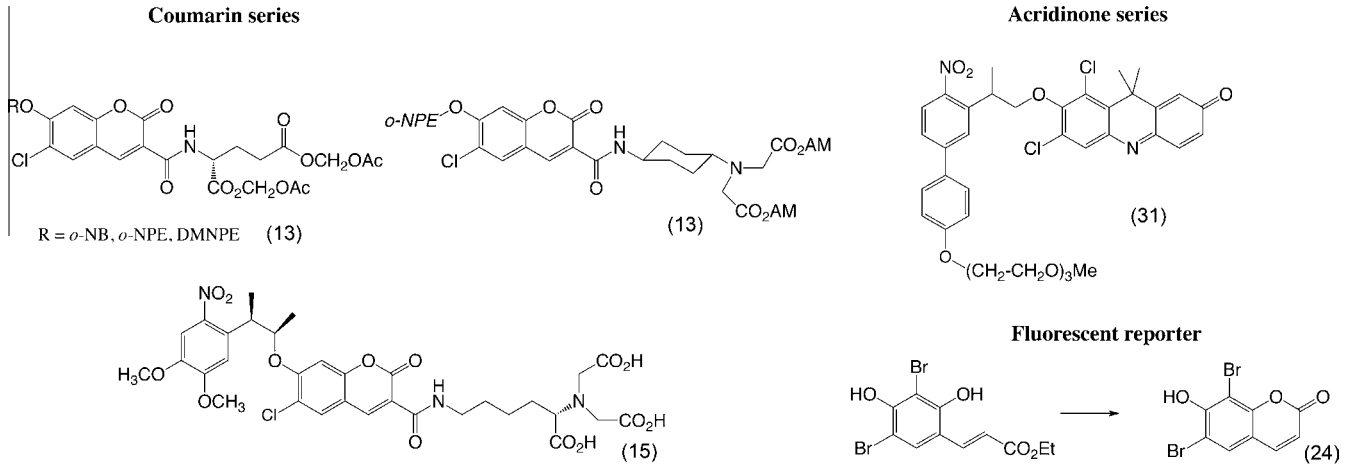


Figure 3. Fluorophores which have been subject to two-photon uncaging.

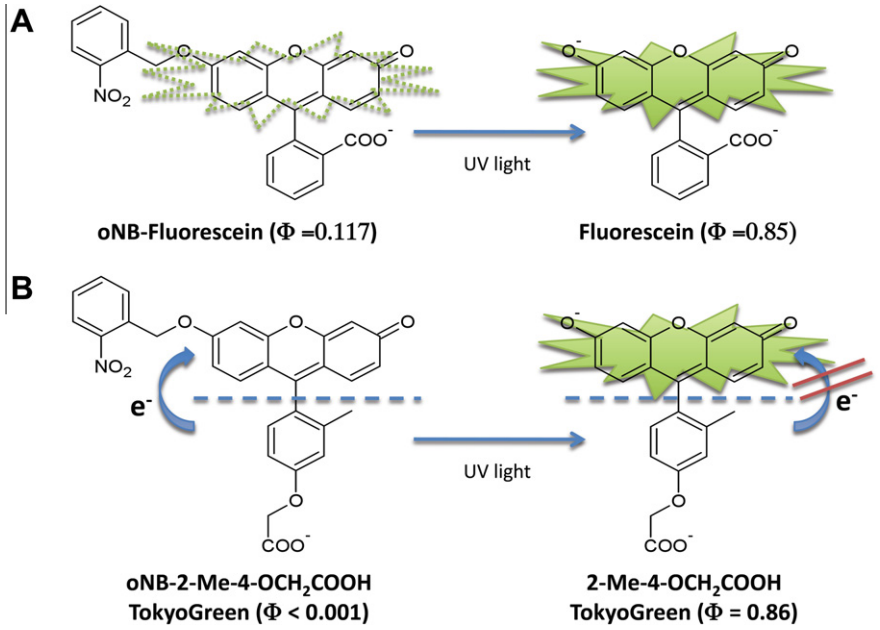
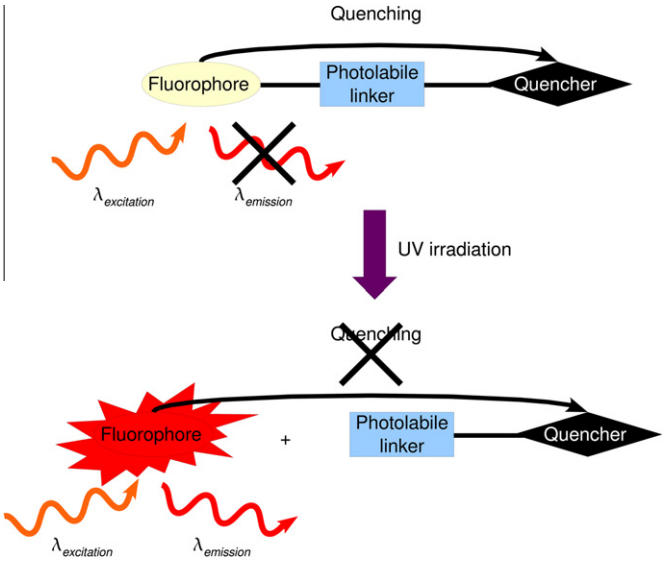


Figure 4. (A) The photolysis reaction of *o*-NB-fluorescein. (B) The photolysis reaction of *o*-NB-2-Me-4-OCH_2COOH TokyoGreen. An intramolecular photoinduced electron transfer makes the fluorescence before photoactivation 100 times lower than that of the *o*-NB-fluorescein.[14]

is no longer favoured. Indeed caged 2-Me-4-OCH_2COOH TokyoGreen is 100 times less fluorescent before photoactivation than mono *o*-NB fluorescein.

Photo-annihilation of a photoinduced electron transfer quenching system seems to be a promising way in the search for new photoactivatable fluorophores.

2.2.2.3. Photolabile linkers for quenched FRET. FRET-based tests using small fluorescent probes are well described for the analysis of the activity of proteases and esterases, see, for example, the seminal article published on the control of the activity of HIV protease using a fluorogenic peptide substrate.[39] Knowing that these enzymes generate a cleavage of their substrates, FRET was a rather obvious choice for the design of fluorescence-based tests. Extension of this concept to the deciphering of other cellular events required the design of a different strategy to allow the simultaneous triggering of protein activity together with fluorescence. Such concept was achieved by modifying a fluorescently-labelled protein, through a linker containing a photocleavable group that can suppress the protein activity and quench the fluorescence (Scheme 2). This concept was applied to a Smad2 protein construct via expressed protein ligation and using a 4,5-dialkoxy-2-nitrobenzyl derivative for the photolytic reaction.[40a] More recently, the same concept was used to target mitochondrial compartments using MLS peptides (mitochondrial localization sequences) that are fluorescently labelled and connected to a quencher through a photolabile linker.[40b] Photolysis produces a sharp increase of fluorescence which can be quantified by flow cytometry. On a similar principle are based SNAP-tag reactive photoactivatable and photo-convertible fluorophores which have recently been described by Johnsson and co-workers. In these systems a SNAP-tag reactive fluorophore is coupled via a photocleavable linker ether to a FRET quencher or to a fluorescent FRET acceptor.[40c]



Scheme 2. FRET-based quenching system controlled by photochemical cleavage of a linker.

2.2.3. Photoswitchable probes

Caged fluorescently-labelled proteins are of primary interest for biological imaging, in particular for the analysis of protein dynamics[41,11] and super-resolution multicolour fluorescence microscopy.[16] Although, the light-directed activation allows a spatio-temporal control of the protein activity, the method relies on a single activation process and might also generate toxic side-products. To overcome these limitations, photochromic systems such as the nitrobenzospiran derivatives[42] have been developed. They allow an optical switching at appropriate wavelengths between a dark (colourless spiro form) and a colourful state (fluorescent open merocyanin form). A reversible FRET imaging method named OLID-FRET (Optical lock-in detection) has been developed accordingly, using this photo-convertible system[43] where the energy transfer reaction from a donor molecule (FITC-Phalloidin or GFP) can be reversibly governed allowing to increase substantially and unambiguously the sensitivity of FRET detection. The attachment of this photoreversible system to a green fluorescent-alkylguaninetransferase (GFP-AGT) fusion protein using a SNAP-tag approach allowed to extend this methodoly to in vivo imaging studies.

2.3. In vivo fluorescent protein labelling using small tags

A series of fusion proteins covalently modified with fluorescent probes have been exhaustively described in the literature[44] complementing judiciously the panel of GFP's and their genetic constructs by allowing to attach fluorophores with improved biophysical properties over the GFP's. Although many different tags have been described in the literature, only a very few have been demonstrated to allow intracellular imaging.[45] Nevertheless, as for the GFP's, these constructs will generate proteins with highly increased molecular weights over the native protein, emphasizing again the need for small tags and photoactivatable fluorophores.

2.3.1. Protein bioconjugation

The classic protein bioconjugation uses the chemical characteristics of the different functions present in the side chains of the canonical natural amino acids. Most protein modification methods are therefore exploiting the nucleophilic character of amino acid side chains like the thiol group of cysteines or the amino function of lysines. This approach has been successfully applied for the study of actin dynamics using a photoactivatable fluorescently-labelled protein by site specific cysteine coupling with a thiol-reactive caged carboxyfluorescein.[11]

However, because proteins usually possess multiple copies of these residues, specific labelling might be difficult to achieve. Therefore several strategies have been developed to introduce unique functionalities into proteins that are chemically orthogonal to the 20 proteogenic amino acid side chains.

2.3.2. Incorporation of nonnatural amino acid into proteins

The incorporation of unnatural amino acid, allows to introduce site specifically into a protein, most functionalities.[46] This was accomplished by chemically aminoacylating a nonsense suppressor tRNA ($tRNA_{CUA}$) with the desired unnatural amino acid and by adding the aminoacyl tRNA to a transcription/translation system along with the gene of interest harbouring a TAG mutation at the target site. This technique allows to incorporate, site specifically, an unnatural amino acid with either unique spectroscopic or biophysical properties (e.g., fluorescent, photoactivatable amino acids), or functional groups that possess unique chemical reactivity orthogonal to those found in natural biomolecules. In the latter case, bioorthogonal reaction can be performed to site specifically label the protein of interest with the desired tag, as a typical example incorporation of an alcyne- or an azido-containing amino acid allows a subsequent specific Cu-free intracellular click-type reaction[47] to attach any probe to the protein. Although this methodology is quite powerful, it requires to be improved in the future, to allow a larger production of protein and the routine use in mammalian cells.[48]

2.3.3. Genetically encoded small peptide tags

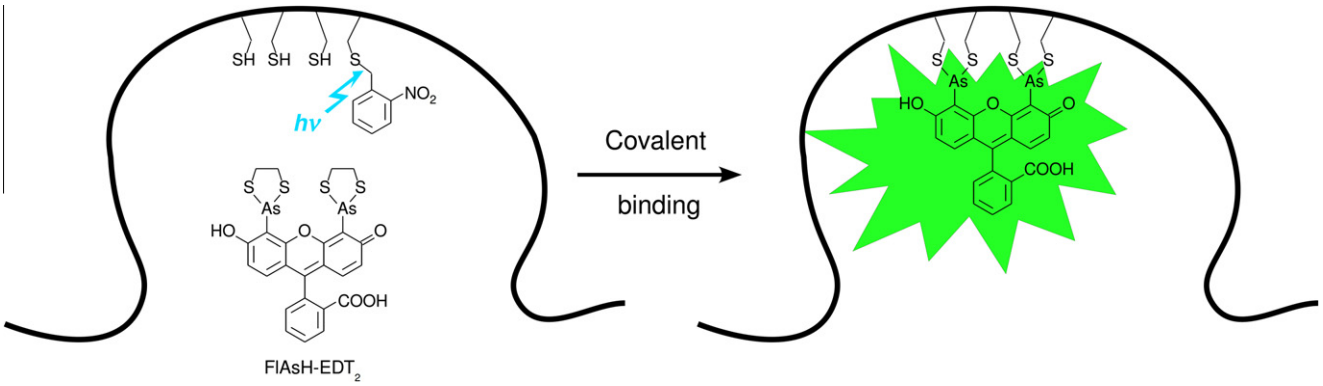
A specific peptide sequence, that can be genetically encoded into the protein of interest, can generate new functionalities for selective chemical modification. The specific recognition of a pro-fluorophore by a peptide motif to generate a fluorescent signal after reversible complex formation opens the way for specific labelling of recombinant proteins in vivo.

This methodology has first been described by Tsien and co-workers by developing the bis-arsenial-functionalized fluorescent dyes (FLAsH-EDT[49] and ReAsH-EDT[50]). They where able to show that a tetracysteine motif CCXXCC, when situated in a hairpin structure, reacts selectively with bis-arsenite derivatives such as FlAsH and ReAsH, leading to a dramatic enhancement of fluorescence upon binding to this genetically encoded small motif. An interesting description of a photochemical control of fluorescence activation through protein FlAsH labelling has been described recently by incorporation of a caged cysteine into the tetracysteine motif of the protein sequence.[51] (Scheme 3)

Similar to FlAsH and ReAsH, Schepartz and co-workers have recently reported an cell permeable and noncytotoxic bisboronic acid rhodamine-based dye (RhoBo).[52] They were able to show an enhancement of fluorescence after binding of this bisboronic derivative to a SSXXSS-like peptide segment.

Besides the labelling of a pro-fluorophore by a peptide motif to generate a fluorescent signal, a small peptide motif can also be used to chemoselectively modify a protein of interest. The conventional His tag (–$(His)_n$–/Ni(II)–NTA) pair was used for fluorescent labelling of a protein on a cell surface.[53] This peptide motif was also used to enhance a specific fluorescence in the presence of Hist tagged protein in an in vitro context. Therefore a hydroxycoumarin fluorophore was joined via a linker to a metal–NTA moiety which forms a weak intramolecular complex, thereby quenching the fluorescence. In the presence of His tagged proteins, the fluorophore was displaced from his intramolecular complex leading to a dramatic enhancement of fluorescence.[54]

Nevertheless, these approaches still suffer from the relatively low stability of the reversible complex formed between the label and the tag.[55] To address this question, tris–NTA probes have been developed displaying a much higher recognition affinity for the 6-His or 10-His tags.[56] Finally, potential cellular toxicity can be avoided by replacing Ni with nontoxic Zn-conjugates.[57] Altogether, these probes to be useful require to be cell permeable for intracellular applications, a first step in this direction was undertaken by developing a new cell permeable Ni–NDA derivative able to



Scheme 3. Photochemical control of a fluorescence activation by a caged peptide recognition motif.[51]

penetrate and eventually regenerate the Ni–NTA recognition motif within HeLa cells.[14]

3. Conclusion

The search for an ideal small pro-fluorescent probe that possesses all the desired physico-chemical properties for a specific cellular fluorescence activation in a spatial and time-controlled manner remains still a major challenge. New techniques for bioorthogonal protein labelling that exhibit fluorogenic properties have been described recently[58,59] allowing to extend the tool box for controlled fluorescence activation on proteins. Concerning the smaller size probes, whilst the uncaging approach and especially the two-photon activation satisfies the spatio-temporal control of the fluorescent signal, the only small size fluorogenic tags developed for protein labelling are the tetracysteine tag and more recently the tetraserine tag (see Section 2.3). Nevertheless, these tags clearly recognize a restricted number of fluorophores whose biophysical properties are not necessarily optimal, therefore the search for new small fluorogenic tags that can be genetically encoded and which could recognize a large panel of fluorescent probes, including caged fluorophores, represents a future challenge for bioorganic chemist in the field of cellular imaging.

Acknowledgements

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