

Photoactive molecules for applications in molecular imaging and cell biology

Qing Shao and Bengang Xing*

Received 26th November 2009

First published as an Advance Article on the web 17th May 2010

DOI: 10.1039/b915574k

Photoactive technology has proven successful for non-invasive regulation of biological activities and processes in living cells. With the light-directed generation of biomaterials or signals, mechanisms in cell biology can be investigated at the molecular level with spatial and temporal resolution. In this *tutorial review*, we aim to introduce the important applications of photoactive molecules for elucidating cell biology on aspects of protein engineering, fluorescence labelling, gene regulation and cell physiological functions.

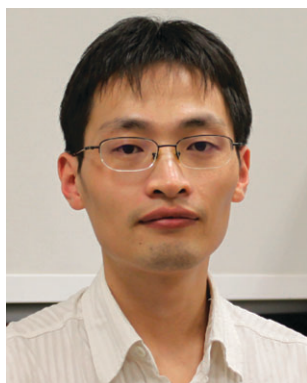
1. Introduction

Cell biology involves the study of cellular components, elucidation of cellular functions and monitoring of relevant biological pathways based on the structure of constituent molecules. Cell structures exhibit dynamic and complex spatial and temporal organization properties. How to precisely control the dynamic properties of cellular functions, and monitor the complicated biological processes at a desired time or location in intact cells or organisms is of fundamental necessity to fully understand the intrinsic mechanism of biological systems at a molecular level. One appealing approach capable of providing such information is through the photo-chemical regulation of

active biomolecules, by which the activation process can be easily manipulated by a beam of light that can be precisely directed to the targets in the cell with a high spatial and temporal precision without significant perturbation of adjacent cellular functions.^{1–4}

There are two distinct strategies commonly employed for applications of photoactive technology in biological systems based on their mechanisms to initiate light-responsive biological reactions. The first one is usually referred to as photoactivatable or photocaged molecules, which involves the deactivation of a particular biologically active molecule through the covalent installation of a photolabile protecting group at a critical position. Generally, biological molecules modified with photolabile groups in the active sites stay inactive before photolysis. However, upon brief light illumination, the protecting groups are cleaved and the caged biomolecules are released irreversibly, thereby recovering the intrinsic bioactivity.

Division of Chemistry & Biological Chemistry, School of Physical & Mathematical Sciences, Nanyang Technological University, Singapore. E-mail: bengang@ntu.edu.sg; Fax: +65 67911961; Tel: +65 63168758



Qing Shao

Qing Shao was born in Jiangsu, China in 1978. He received his BE degree (2001) and MS degree in Organic Chemistry (2004) from Suzhou University under the direction of Prof. Qi Shen. After that, he joined a pharmaceutical company, Basilea Pharmaceutica China. In 2009, he went to Nanyang Technological University for graduate studies under the supervision of Prof. Bengang Xing. His research field is mainly focused on the construction of novel optical imaging probes and their applications in bioassays.



Bengang Xing

Bengang Xing received his BS degree in 1994 from Xinjiang Normal University. He obtained his MS degree in chemistry in 1997 from Guangxi Normal University under the direction of Prof. Liang Hong and his PhD from Nanjing University in 2000 under the supervision of Prof. Tang Wenxia. After two years working in Prof. Xu Bing's group as a research associate at the Hong Kong University of Science and Technology, he moved to the Molecular Imaging Program at the University of California, Los Angeles and Stanford University to do post-doc research with Prof. Rao Jianghong. He started his independent research at the School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore in December 2005. His current research interests include fluorescent imaging, chemical biology and bio-nanotechnology.

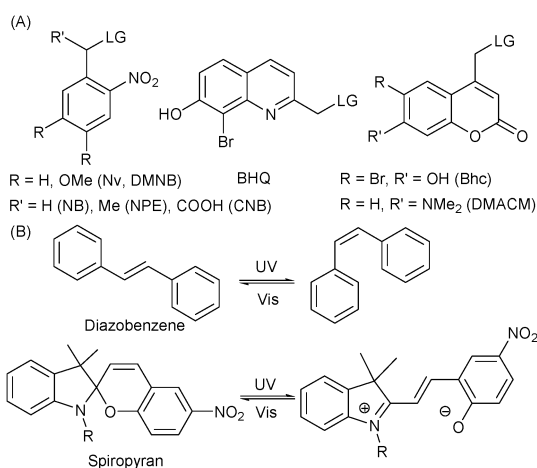


Fig. 1 (A) Molecular structures of selected photolabile protecting groups; (B) light sensitive molecules with photoswitchable structures.

Fig. 1 shows a list of some well-known photocaged protecting groups. Among them, 2-nitrobenzyl (NB) and its derivatives together are by far the most commonly used moieties in biological systems. Up to date, photocaged technology has been extensively utilized as a powerful tool to monitor biological processes and study their functions in well-resolved space and time resolutions.^{1–5}

The other commonly used strategy based on light sensitive molecules such as spiropyran^{1,6} or diazobenzene^{1,7} has also been constructed and applied to control biomolecular activities by illumination with light of different wavelengths. Unlike the irreversible photorelease in the photocaged strategy, these types of light-sensitive molecules undergo reversible photoswitches, leading to conformational or structural exchanges between “active” and “inactive” states upon irradiation with UV or visible light (Fig. 1B). All these photoactivatable or light-sensitive molecules are easily synthesized and can be efficiently installed into the structure of targeted biomolecules. More importantly, they offer the great advantage to precisely monitor the spatiotemporal dynamics of biological processes with high specificity and transient light-sensitivity.

In this tutorial review, we will primarily focus on the latest advances in the biological applications of synthetic photoactive molecules in cell biology, with an emphasis on those studied through molecular imaging. We will also introduce the applications of photoactive molecules in protein engineering, gene regulation and cellular physiological functions. At the end of this review, we will offer the perspectives and discuss the future challenges and promises of photoactive technology in biological systems. Several excellent reviews regarding the design of photoactive molecules,¹ photochemical mechanisms,⁴ biological applications^{2,3,5} and experimental strategies (such as light source, laser instrumentation or delivery techniques)^{2,3} have been summarized previously and, therefore, will not be discussed in this review. Other applications based on naturally occurring light-responsive macromolecules, such as light-activated fluorescent proteins or plant photoreceptors, and a vast number of other applications based on caged small biomolecules like ATP will not be extensively introduced because of the limited space.

2. Photoactive molecules for protein engineering

Understanding protein function is essential in cell biology because proteins play central roles in maintaining cell life. The biological functions of proteins rely on their three dimensional structures and intrinsic physical-chemical properties. One direct strategy for the precise control of biological processes is to chemically engineer photo-sensitive groups into the active sites of protein structures. Upon the direct delivery of modified proteins into living systems, study of protein functions and their dynamic properties can be easily achieved through photoactivation.

One practical approach to constructing photoactive proteins is through the chemical modification of the active side chains in amino acids. For example, Bayley and Lawrence independently introduced caging groups to a subunit of cAMP-dependent protein kinase (PKA) through direct alkylation of cysteine residues with surprisingly high efficiency.⁸ However, this random introduction is extremely dependent on the property of activation loop and reactivity of modification reagents and is not applicable to proteins with multiple functional domains. One rational design to overcome this drawback is to develop a chemo-enzymatic process for site-specific protein labelling, mostly by using 4-hydroxyphenacyl group or NB derivatives.⁹ This process involved the sulfur alkylation of a reactive thiophosphate, which was introduced enzymatically into a protein at a critical residue, usually serine, threonine or tyrosine, and was responsible for a dramatic alteration of protein function.

Selective and covalent protein labelling with small synthetic fluorescent probes is another strategy for studying protein functions and tracking the dynamics of cellular events in real-time. Such protein labelling strategies can be easily obtained through specific chemical reactions based on recognizing peptide motifs such as SNAP-tag, His-tag and tetracysteine. Labelled with caged fluorophores, proteins can be used for biological studies where the fluorescent signal is spatially and temporally controlled. For example, Specht and co-workers used 3-(4,5-dimethoxy-2-nitrophenyl)but-2-yl (DMNPB) caged coumarin derivatives to label His-tagged proteins through recognizing the His-tag motif by Ni-nitrilotriacetic.¹⁰ Similarly, Johnsson and his group developed a general approach for covalent labelling of fusion proteins based on the irreversible reaction of DNA transferase (AGT or SNAP-tag) with *O*⁶-benzylguanine (BG) derivatives. The 1-(2-nitrophenyl)-ethyl (NPE) caged BG derivatives were synthesized and their activity towards SNAP-tag fusion proteins was regulated through photolysis.¹¹ Very recently, the group of Cropp genetically encoded NB-caged cysteine into proteins for FIAsh labelling.¹² Photolysis of caged protein resulted in the formation of a tetracysteine motif in yeast cells, which was specifically targeted by FIAsh or other biarsenical derivatives, and generated distinctively fluorescent protein-bound complexes. They found the light-mediated labelling was efficient and the photoactivation of caged cysteine tags was nearly quantitative, providing the possibility for multicolor imaging of living systems (Fig. 2).

The photolabelling reagents have shown great promises for real-time observation of cellular events *in vitro* and in living

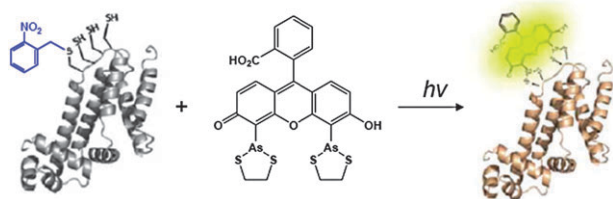


Fig. 2 Photoactivated protein labelling through FIAsH. Adapted with permission from ref. 12. Copyright 2009, Elsevier.

cells with fluorescence imaging technique. Compared with fluorescence imaging modality, bioluminescence imaging (BLI) exhibits higher sensitivity and lower background, thereby providing a noninvasive approach for the study of ongoing biological processes in live animals, such as tumour growth, drug response and gene expression. Tracking the dynamic properties of these events also desires the spatiotemporal control of bioluminescent signals, which can now be achieved through photoactive imaging probes. Xing and co-workers recently developed a set of photoactivable bioluminescent probes for detecting firefly luciferase (fLuc) expression *in vivo* (Fig. 3).¹³ The bioactivity of D-luciferin was fully deactivated as the 6-hydroxy group was blocked by NB caged groups. Illumination of photocaged substrates resulted in the recovery of D-luciferin activity. The generated bioluminescence was capable of imaging luciferase expression in living mice. Therefore, the combination of photocaged technology and fLuc report gene system provided the opportunity for real-time monitoring of the dynamics of individual cellular functions *in vitro* and *in vivo*.

Targeted protein modification can also be obtained through nonsense suppression mutagenesis and expressed protein ligation,^{14,15} which are more useful in protein engineering due to the predictability and reproducibility of this technique. With these approaches, photocaged amino acids can be incorporated into natural proteins in a site-specific manner.

Schultz and co-workers developed orthogonal tRNA-synthetase pairs to selectively incorporate unnatural amino acids into proteins with high efficiency and fidelity.¹⁶ By this means, caged cysteine was genetically encoded in caspase-3 in response to the amber nonsense codon TAG, and the protease activity could be controlled by photoactivation. They also improved this methodology to photoinitiate the phosphorylation of Pho4 by using 4,5-dimethoxy-2-nitro-benzyl (DMNB) as a caging group which was sensitive to less toxic blue light.¹⁷ The Pho4-GFP mutant was genetically encoded with DMNB-serine and localized in the nucleus. Laser photolysis removed the protecting group and resulted in the formation of wild-type

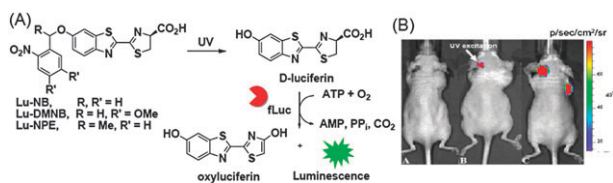


Fig. 3 (A) Mechanism of photoactive bioluminescence; (B) imaging of fLuc activity in living mice upon illumination. Copyright 2009, Royal Society of Chemistry.

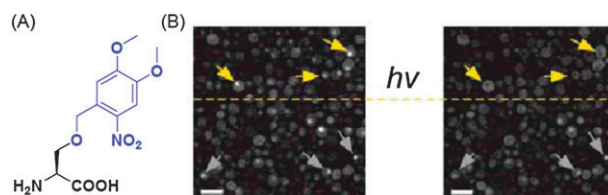


Fig. 4 (A) Structure of DMNB caged serine; (B) photolysis of Pho4-GFP mutant generates a strong decrease of fluorescence in nucleus. Copyright 2007, Nature Publishing Group.

Pho4-GFP, which was then phosphorylated in the presence of extracellular inorganic phosphate and translocated to the cytoplasm. This method could be used to monitor the kinetics of protein export in real-time by measuring the fluorescence decrease in the nucleus (Fig. 4). Similarly, a DMNB-caged lysine was also incorporated in bacterial and mammalian cells with a pyrrolysyl-tRNA/PyIRS pair, thus establishing an *E. coli*-mammalian “shuttle” system which can be helpful for controlling the chemical composition of mammalian proteins.¹⁸

Using the same strategy, another photoactivable Taq DNA polymerase was recently developed through the introduction of NB-caged tyrosine at position Y671, which allowed the spatiotemporal control of DNA polymerization *in vitro* (Fig. 5).¹⁹ This light-responsive NB protected tyrosine was also specifically installed in the catalytic site of Cre recombinase and used to inhibit its activity. Exposure to UV light resulted in the removal of the caging group and enabled DNA recombination *in vitro* and in a single mammalian cell.²⁰

Endo and Majima achieved the photochemical regulation of caspase-3 activity by incorporating a 2-nitrophenylglycine residue into a caspase-3 precursor through an artificial four-base codon approach.²¹ Upon irradiation, the intrinsic caspase-3 activity could be recovered through pre-designed site-specific cleavage.

Muir *et al.* developed a caging strategy for phosphoproteins using expressed protein ligation.¹⁴ As a proof of concept, an NPE-caged phosphopeptide was ligated to Smad2, a cellular signaling protein activated by phosphorylation events. The caged protein was then allowed to study the localization in nucleus before and after irradiation. By taking advantages of protein phosphorylation and photolysis, this approach provides the possibility of investigating the molecular photocontrol of protein functions with living cell imaging technique. Later, the same authors utilized the similar protein ligation strategy to construct caged proteins where the uncaging efficiency was

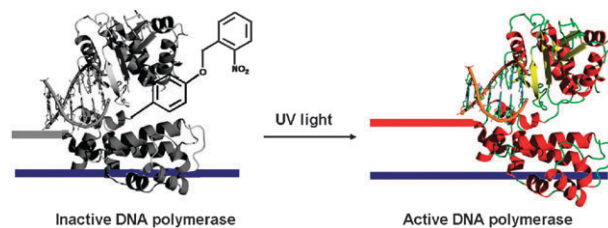


Fig. 5 Photoactivated DNA polymerase. Adapted with permission from ref. 19. Copyright 2009, Wiley-VCH Verlag GmbH & Co. KGaA.

directly correlated to fluorescence signal.¹⁵ In this approach, the ligation and photorelease molecule was designed to combine a *o*-nitrobenzyl group for photocleavage, a 2-aminoethanethiol group for chemical ligation and a carboxylic acid tether for further conjugation with other amine containing functional groups such as fluorescent Texas Red-X (TR) or lipidic palmitoyl group (Palm) (Fig. 6B). The model protein was designed as enhanced green fluorescent protein containing nuclear localization signal (EGFP-NLS) at the C-terminus with additional utilities for facile fluorescent labelling and monitoring. Upon the ligation between the photocaged peptide and α -thioester modified protein EGFP-NLS (Fig. 6A), the fluorophore labelled protein was able to undergo an intramolecular fluorescence resonance energy transfer (FRET) from EGFP to TR. Irradiation with light led to the photorelease of a controlled amount of cleaved product inside living cells. In addition, the localization of EGFP-NLS could be photochemically controlled through the incorporation of a lipid. As expected, Palm labelled proteins specifically targeted cell membrane and diffused in the cytoplasm and nucleus with distinctive fluorescence enhancement after photocleavage of the lipid moiety (Fig. 6C).

Lawrence and co-workers introduced a clever method for the light-mediated liberation of Src kinase activity.²² They built up a series of peptide-based kinase inhibitors which were bivalent and embedded with photocleavage groups. Photolysis split the peptides and destroyed the inhibitory actions, with the concomitant recovery of kinase activity (Fig. 7). Geierstanger *et al.* demonstrated the photolytic cleavage of ¹⁵N-labelled *o*-nitrobenzyl tyrosine incorporated into human fatty acid synthase *in vivo*, which were employed to facilitate the introduction of NMR label at specific amino acid residues without altering the protein sequence and binding surface.²³ This research will be helpful to establish a new avenue for the site-specific labelling of proteins at individual residues and enabling NMR studies of large proteins that cannot currently be studied by traditional methods.

Most of the light-regulated biological processes involve the installation of irreversibly photolabile groups into protein structures. Actually, similar functions can also be obtained

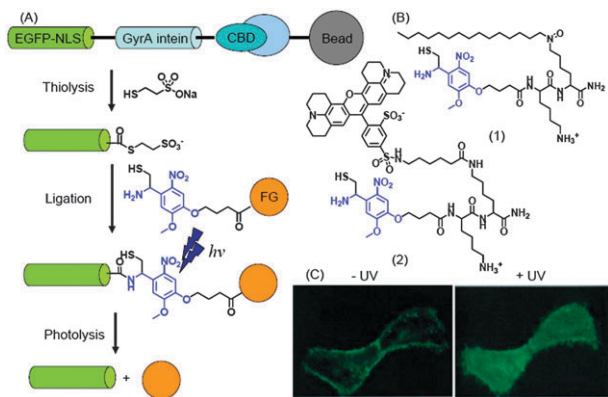


Fig. 6 (A) Mechanism of expressed protein ligation; (B) structures of functional Palm (1) and TR (2); (C) photocontrol of subcellular localization of Palm labelled protein. Adapted with permission from ref. 15. Copyright 2005, Wiley-VCH Verlag GmbH & Co. KGaA.

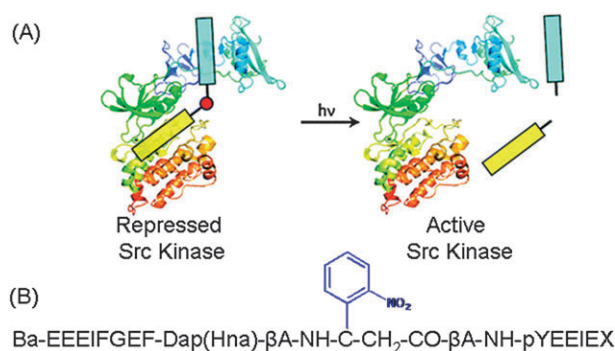


Fig. 7 (A) Photocleavable caged protein equivalent; (B) selected photosensitive Src inhibitor. Copyright 2008, American Chemical Society.

by modification with reversibly photoswitchable moieties. Meijberg and Feringa developed a molecular valve by attaching a synthetic spiroopyran-merocyanine compound to a channel protein.⁶ This valve was embedded in a membrane and controlled by irradiation, that is, opened by the charge separation process as a result of 366 nm irradiation and closed in response to visible light above 460 nm (Fig. 8A). With the light-gated property, this system could be used to deliver biomolecules or drugs in a light controlled manner.

Isacoff and Trauner presented another optical switch to achieve photochemical control of an ion channel in living cells. In this approach, GluR6, an ionotropic glutamate receptor, was engineered by site-specific attachment of a tethered glutamate derivative containing a photoswitchable diazobenzene moiety to the ligand binding domain clamshell. Irradiation at 380 nm generated the *cis* form of diazobenzene and opened GluR6. Irradiation at 500 nm triggered the closure of GluR6 (Fig. 8B). Repeated light stimulation would enable the allosteric control of an ion channel in a time-resolved process.^{7,24}

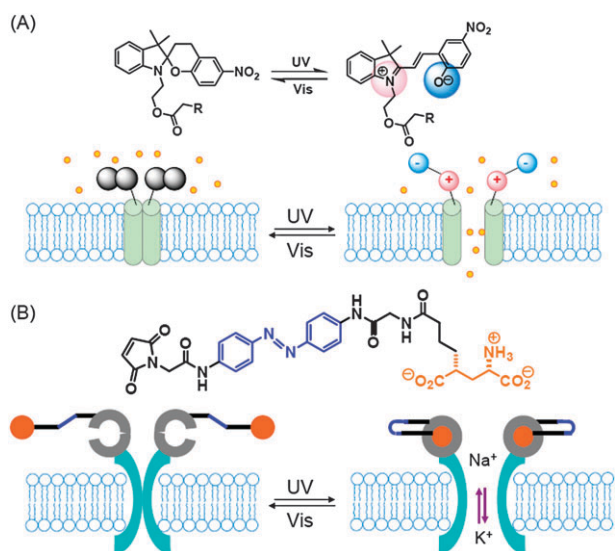


Fig. 8 (A) Schematic presentation of photoswitchable molecular valve; (B) schematic presentation of photoswitchable ion channel. Adapted with permission from ref. 7. Copyright 2006, Nature Publishing Group.

Similarly, the photoswitchable diazobenzene moiety was also incorporated into one subunit of α -hemolysin at a single cysteine residue (Cys117) to construct the protein pore in a lipid bilayer.²⁵ The photoinduced *trans-cis* isomerization of structure modified protein pore could be used as a “nanoreactor” for the observation of small-molecule chemistry in water at the single-molecule level.

Significant advances have been achieved in developing the light-mediated peptides and proteins for precise regulation of protein functions and biological activities.^{1–4} However, some technical challenges still need to be addressed before their regular applications in living systems. For example, some of the photoactive molecules lack stability and chemical installation of the photolabile protecting groups may not completely block the peptide or protein activities, which could lead to little differences between the free molecules and their photo-labelled counterparts in biological studies. In addition, site-specific peptide or protein labelling remains the principle barrier for the utilization of photoactive technology in living cells. The development of novel light sensitive molecules and new caging strategies with improved photophysical and photochemical properties are needed for further biological applications.

3. Photoactive molecules in gene regulation

Gene expression deals with the conversion of genetic information from DNAs into RNAs and proteins, allowing the generation of desired proteins and increasing the versatility and adaptability of living systems. Chemical approaches for regulating gene expression within the living cells and organisms have offered great opportunities for elucidating complex biological processes at the molecular level. Although proteins have been the traditional targets to achieve the photoregulation of biological processes, photoactive techniques for the targeting expression of genetic materials in cells or organisms with spatial and temporal resolution provide a more stable and direct way to control biological functions and have proven extremely successful in biological studies.²⁶ Technically, spatiotemporal regulation of genetic functions can be obtained through the installation of photoactive moieties onto the phosphorus backbone or the nucleotide base in both DNA and RNA fragments. In this section, we will mainly focus on the approaches for the photochemical control of genetic processes, especially those dependent on the formation of functional oligonucleotides upon photolysis.

3.1 Photoregulation of DNA function

Haselton and co-workers first demonstrated the introduction of photolabile groups into plasmid DNAs coding for luciferase or GFP expression through a direct alkylation with the 4,5-dimethoxy-2-nitrophenyl diazoethane complex.²⁷ The DMNB-caging groups were introduced statistically at the phosphate backbone in an unselective manner. After the transfection of the purified photocaged plasmids into rat skin cells or HeLa cells, these caged plasmids did not express target proteins. However, the subsequent exposure of transfected skin sites to 355 nm laser light induced the expression of luciferase or GFP respectively in a dose-dependent manner. This photocage technology could be useful to protect plasmids

from methylation and/or degradation, considering DNA methylation generally turns off gene expression. Although the efficient phosphorus backbone photocaging was elegant due to its simplicity of preparation, this method was less feasible to obtain the precise control of a specific nucleic acid labelling, which was necessary for the study of gene expression at the molecular level.

The group of Heckel introduced a widely applicable strategy for the site-specific control of oligonucleotides, with the smart idea of introducing a caging group as a temporary mismatch to block Watson–Crick base pairing, which is the nature of nucleic acids in the storage and flow of genetic information.²⁸ They used this method to activate gene transcription through photolysis. In this work, caged thymidines were incorporated into DNA oligodeoxynucleotides at pre-designed positions and prevented T7 RNA polymerase from recognizing the promoter region, due to a severe perturbation of the local duplex structure resulting from the mismatch. The transcriptional activity was blocked in this way and fully activated upon photolysis. Thus, the transcription can be controlled with only one or two caged nucleotides in a light-dependent manner. A similar design was also developed to photochemically control the DNA-protein interactions for restriction endonucleases by installation of one single 6-nitropiperonyloxymethyl protected thymidine into a DNA sequence.²⁹ The photocaging group prevented enzyme cleavage when located close to the recognition site and dramatically decreased enzymatic catalytic properties. However, upon the photochemical removal of the photolabelling group, the enzyme activity was recovered completely (Fig. 9).

An alternative strategy to photoregulate DNA polymerase reaction was developed by the group of Komiyama, who introduced a photoswitchable diazobenzene moiety into an oligodeoxynucleotide modulator, which was then bound to a template DNA in the primer extension region.³⁰ The planar *trans*-azobenzene stacked favorably within the duplex structure, which was not subject to T7 DNA polymerase and blocked the DNA elongation at its 5'-end (Fig. 10). Under UV irradiation, the nonplanar *cis*-isomer weakened the base-pairing and induced the polymerase to knock off the modulator, resulting in the formation of a full-length strand.

Similarly, light regulation can also be accomplished to control thrombin activity by activating/deactivating the thrombin aptamer with azobenzene-modified nucleic acid

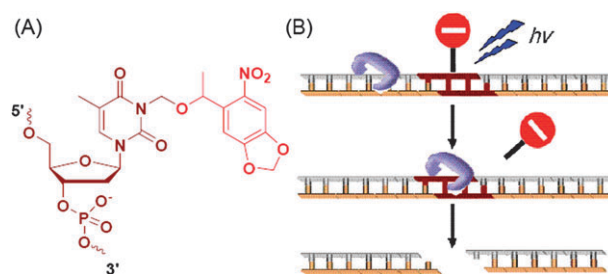


Fig. 9 (A) Structure of caged DNA; (B) scheme of light-triggered DNA cleavage by restriction endonucleases. Adapted with permission from ref. 29. Copyright 2009, Wiley-VCH Verlag GmbH & Co. KGaA.



Fig. 10 Photoregulation of DNA polymerase reaction. Adapted with permission from ref. 30. Copyright 2000, Wiley-VCH Verlag GmbH & Co. KGaA.

modulators.³¹ Thrombin activity can be inhibited through the specific binding of the DNA aptamer, which was activated *via* dehybridization from the complementary DNA sequence induced by UV illumination. With this probe, people could control the clot formation in a site-specific manner with submillimetre resolution by local visible light treatment. This approach might be helpful for biomedical and pharmaceutical applications (Fig. 11).

3.2 Photocontrol of RNA processing, degradation and translation

The primary transcripts of mRNA undergo several processing steps, such as capping, polyadenylation, splicing or nuclear export, to become mature and functional before they are transported to the cytoplasm and further translated into proteins. These biological processes are of great importance to functionalize RNA structure and optimize RNA recognition that profoundly influence their following transcription or translation. The incorporation of photolabelling groups with RNA molecules can provide a feasible and straightforward approach to understanding the processing mechanisms and achieving the spatiotemporal control of related biological functions. The first photoactive caging RNA molecule was developed by the group of MacMillan.³² In this work, NB-caged groups were introduced specifically to 2'-OH groups of adenosine, which was incorporated in substrate RNA sequences and arranged essential to the ribozyme activity. As expected, the caged substrate RNA was no longer sensitive to the ribozyme, whereas the photocleavage of caging groups fully restored the activity and would be useful to probe RNA reactivity and dynamics. They also used this technique to

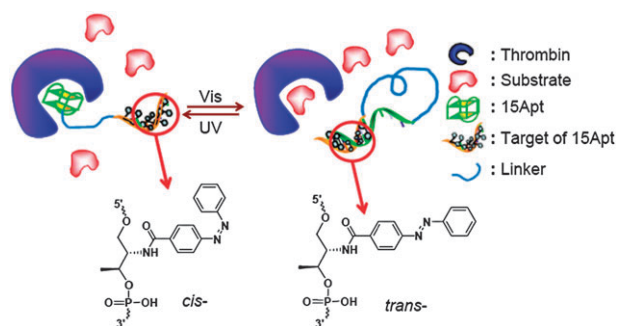


Fig. 11 Illustration of strategy for the photoregulation of thrombin activity using reversible photoswitch azobenzene-aptamer probe. Copyright 2009, National Academy of Sciences, USA.

study spliceosome-catalyzed pre-mRNA splicing, in which the noncoding intron sequences were removed and the coding exons were stitched together.³³ With this strategy, they could identify the spliceosome assembly independent of its catalytic reaction, which was normally difficult to obtain using a normal approach.

Targeted RNA degradation helps cells to defend against potentially dangerous double-stranded RNA molecules. In this process, small interfering RNA (siRNA) molecules are incorporated into an RNA-induced gene silencing complex (RISC), which then discards one strand of the siRNA duplex with a nuclease and targets a foreign RNA molecule complementary to the remaining strand, leading to the degradation of target RNA. Controlled RNA interference is particularly valuable for investigating the expression and function of specific genes in cell development.

The group of Friedman developed a light-dependent method to control RNA interference with caged siRNA molecules.³⁴ GFP-targeting siRNA sequences were modified with 4,5-dimethoxy-2-nitrophenyl-ethyl (DMNPE) caging groups to block the siRNA-RISC interaction and prevent the degradation of target mRNA. With an average of 1.4 caging groups per duplex, the caged siRNA was inert and fully activated upon irradiation to interfere with GFP expression, which was quantified by a fluorescent GFP signal. Recently, they went further and improved this method based on the hypothesis that double-stranded precursors of siRNA (dsRNA) would be more sensitive to modification.³⁵ In this study, terminal phosphates incorporated into dsRNA were selectively caged with diazo-DMNPE. The photo cleavage of terminal phosphate modification was much more facile compared with the approach in general nucleobase modification.

One alternative strategy for modulating mRNA degradation is based on the application of antisense oligodeoxynucleotides (asODNs). Hybridization of a specific antisense ODN to a target mRNA results in the formation of mRNA/asODN duplex, which inhibits translation and leads to mRNA degradation through the recruitment of endogenous ribonucleases. Compared with the approach based on siRNA molecules, this new strategy is more advantageous because antisense ODNs are more stable and exhibit higher efficiency in controlling mRNA digestion and regulating gene functions. The group of Dmochowski indicated the photoregulation of mRNA degradation catalyzed by RNase H, which was generally activated by the hybridization of mRNA to an antisense oligodeoxynucleotide.³⁶ A DNA hairpin containing antisense ODN strand was conjugated to its blocking sequence through a photocleavable linkage (Fig. 12). The hairpin stability relative to corresponding RNA/DNA hybrids could be controlled by photolysis. It turned out only a little degradation could be observed with the photocaged hairpin, whereas photoactivation could be achieved within a few minutes and resulted in a 4–10-fold increase in RNase H activity. This antisense oligonucleotide strategy was also useful to photoregulate other biological processes where RNA/DNA hybrids were involved. By the incorporation of a caged thymidine into antisense oligomers, Deiters *et al.* demonstrated a similar design for the precise spatial control of luciferase gene expression in living cells.³⁷

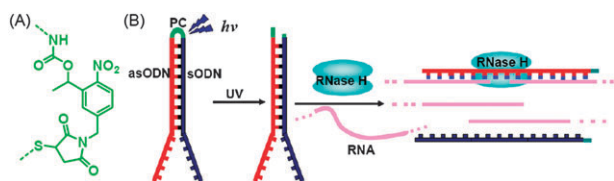


Fig. 12 (A) Structure of photocleavable linker; (B) strategy for regulating RNA digestion by RNase H with photo sensitive DNA hairpin. Adapted with permission from ref. 36. Copyright 2006, Wiley-VCH Verlag GmbH & Co. KGaA.

In addition, the photoregulation of mRNA degradation was also achieved by the incorporation of azobenzene-modified modulators,³⁸ which allowed the formation of RNA/DNA duplex based on *trans* to *cis* photoisomerization of azobenzene moiety and thereby resulted in mRNA digestion by recruiting RNase H.

Translation is the production of proteins by decoding mRNA gene expression. As one important process preceded by transcription in gene expression, translation can be directly targeted to provide a powerful strategy for studying gene function and enabling spatiotemporal gene modulation in living organisms. Okamoto and co-workers presented the first example of the light-mediated regulation of translational activity of GFP-mRNA *in vivo* with photocage technology.³⁹ The photoactive 6-bromo-4-diazomethyl-7-hydroxycoumarin (Bhc-diazo) group reacted with the free phosphoric acids of the GFP-mRNA sequence to yield the Bhc caged phosphate esters, which turned out to be translationally inactive and distributed ubiquitously in zebrafish embryos with remarkable stability. Photoillumination with 350–360 nm ultraviolet light removed the Bhc groups from caged GFP-mRNA and induced the recovery of the translational activity in irradiated regions. With the mRNA backbone caged statistically, this method was rather simple and enabled the precise control of gene expression in living subjects without the necessity to generate any transgenic lines.

A similar study conducted by Chen and co-workers also demonstrated the ability to spatiotemporally modulate gene expression using photoactivable nonsense morpholinos to block RNA translation.⁴⁰ In this method, the synthesized *no tail* (*ntl*) targeting morpholino (*ntl*-MO) was coupled to a fluorescein-labelled inhibitor through a photocleavable DMNB linker and injected in the one-cell stage of zebrafish embryos. As expected, injected embryos without photo exposure developed properly as the *ntl*-MO function was blocked by the inhibitor. However, in irradiated embryos, U-shaped somites were developed instead of the normal rod-shaped notochord, due to the photoactivation of caged *ntl*-MO (Fig. 13). Furthermore, the abnormal notochord could be visualized restricted to a locally irradiated region within a chordamesoderm, thus gene expression could be controlled spatiotemporally with caged morpholinos. This light controlled gene knock-down technology might be helpful to enable targeted genetic perturbations.

In a recent study, the group of Dmochowski used the photoactivated negatively charged peptide nucleic acids (ncPNA) to block ribosomal protein synthesis in living

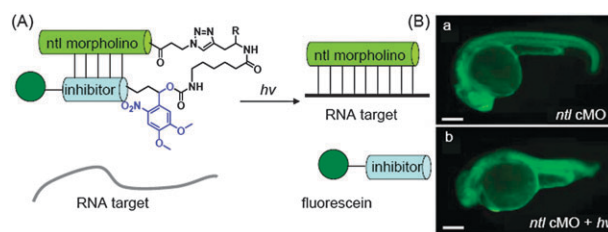


Fig. 13 (A) Scheme of a caged morpholino and its light-triggered hybridization to RNA targets (B); fluorescence of embryo injected with *ntl* cMO (a) and that upon irradiation (b). Adapted with permission from ref. 40. Copyright 2007, Nature Publishing Group.

organisms (Fig. 14).⁴¹ An antisense ncPNA strand targeting initiation codon AUG of zebrafish *chordin* mRNA was conjugated to an RNA sense strand (sRNA) via a photolabile linker, where photocleavage caused the ncPNA/sRNA duplex to be less stable ($\Delta T_m > 20^\circ\text{C}$), resulting in ncPNA binding to target mRNA and turning off gene expression accordingly. The specificity of this method was further tested by visualization of *goosecoid* (*gsc*) expression, one specific genetic marker dependent upon the function of *bozozok*. The regulation of *bozozok* expression could be readily achieved through the photoactivation of caged ncPNA targeting *bozozok* mRNA.

In addition to the active biomacromolecules such as DNA and RNA, some small molecule inducers such as lactose, β -estradiol, ecdysone and doxycycline/tetracycline⁴² are also valid targets for the photochemical control of gene regulation. Relevant biological applications and their advantages in genetic manipulation have been discussed in a recent review.⁵

The photoactive oligonucleotides have shown great potential for gene regulation *in vitro* and in living animals. However, most of the light-mediated gene expression relied mainly on the statistical incorporation of photoprotecting groups on DNA or RNA structures. Precise control of the position and number of incorporated photolabile blocking groups within oligonucleotide structures remains a primary obstacle for their *in vivo* biological studies.^{1–3,26} New synthetic methods and photocaging strategies with consideration of other aspects such as effective cellular delivery, excellent restoration of oligonucleotide activities upon light irradiation and minimum photodamage to the cells should also be further determined in the process of biological investigation.

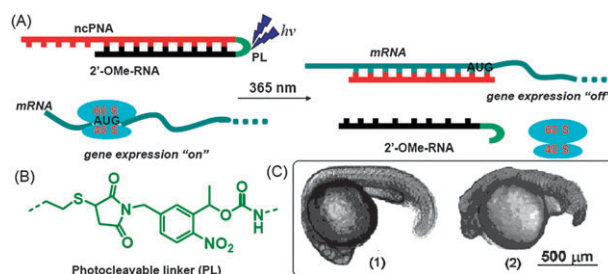


Fig. 14 (A) Controlling gene expression using ncPNA attached to RNA via photocleavable linker (PL); (B) structure of caged linker; (C) transmitted light images of zebrafish embryos. (1) Uninjected; (2) Injected with caged PNA-boz followed by irradiation. Adapted with permission from ref. 41. Copyright 2007, American Chemical Society.

4. Photoactive molecules in the study of cell physiological function

Photochemical approaches have contributed to a better understanding of cell physiology by activating physiological processes at will. Most of these physiological processes including cell adhesion, cell motility and cell cycle are dependent on wide classes of proteins or other recognition molecules, which can be targeted or activated by photoactive probes and therefore the processes will be inactive. However, upon light illumination, the photolysis will remove the photoactive groups and allow the precise control of time-dependent perturbation.

4.1 Photomodulation of cell adhesion and cell migration

In a pioneering work published in 1991, Theriot and Mitchison studied the dynamic behavior of actin microfilaments in locomoting cells.⁴³ Actin was covalently labelled with NPE-caged resorufin fluorophores and copolymerized in stress fibres. The movement and turnover of actin filaments were then investigated by activating and measuring the fluorescence over time. Irradiation with light resulted in a rapid turnover throughout the whole lamellipodium with actin filaments depolymerizing just as rapidly near the periphery and close to the nucleus, which was consistent with the nucleation-release model.

Jacobson and co-workers studied cell locomotion using photoactivated thymosin β 4 (T β 4),⁴⁴ which sequestered actin and inhibited its polymerization activity. The resultant perturbation in cytoskeletal dynamics could affect global cell locomotion. Upon the local photoactivation of T β 4 at the wings of locomoting keratocytes, the specific turning around the irradiated sites was observed. In order to establish the role of cofilin and its mechanistic contributions to cellular motility, one S3C mutant cofilin was covalently modified with α -bromo-(2-nitrophenyl) acetic acid (BNPA), which was microinjected into cells and locally activated upon photolysis. This activated cofilin promoted actin polymerization and acted as a component of the “steel wheel” for cell motility.⁴⁵

Controlled cell adhesion can be obtained through the chemical modification of binding biomaterial. Recently, Del campo and co-workers introduced DMNPB-caged groups into RGD peptides, causing its integrin-binding activity to be blocked.⁴⁶ Photorelease enabled cell attachment to a surface with spatial and temporal control. Another photo-degradable RGD peptide conjugated hydrogel was also recently developed to differentiate the stem cell migration in a 3D photocaged cell culture platform.⁴⁷ With the spatiotemporal manipulation of cell microenvironment, these hydrogels provided a simple and feasible approach to real-time regulation of cellular functions. Compared with other photoactivation systems based on photoswitchable azobenzene moiety,⁴⁸ utilizing caged RGD to trigger cell adhesion exhibited much better results in efficiency and selectivity, although this process was irreversible.

Nakanishi and co-workers built a strategy for positioning single cells onto a glass coverslip through photoactivation under standard fluorescence microscope.⁴⁹ This coverslip was coated with bovine serum albumin (BSA) to prevent cell adhesion. Photocleavage of caged NB group caused

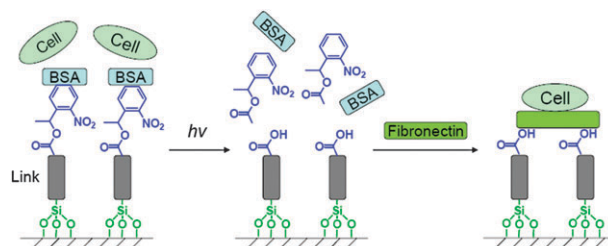


Fig. 15 Photoactivated surface modification for cell adhesion. Adapted with permission from ref. 49. Copyright 2007, American Chemical Society.

dissociation of BSA from the irradiated glass surface and the vacancy selectively adsorbed fibronectin, a cell-adhesive protein, thus controlled cells adhering to the surface (Fig. 15). This photoactive substrate enabled the spatiotemporal control of single cell migration, thereby offering the possibility to study cell-cell interactions microscopically at the single-cell level.

4.2 Photocontrol of the cell cycle

Regulation of the cell cycle is important for the survival of a cell by detecting and repairing genetic damage as well as preventing uncontrolled cell division. Studying the mechanism of the cell cycle is particularly important because cellular cycle dysregulation can result in many diseases such as cancer, heart diseases or neurological disorders. Photochemical modulation has proven useful for investigating the cell cycle with direct control and minimum damage.

Yaffe *et al.* developed a chemical genetics approach using caged phosphoserine peptides to study the general function of 14-3-3 proteins in cell cycle control (Fig. 16).⁵⁰ These peptides worked as inactive precursors with recognition sequence being caged, which could be easily activated upon UV illumination. The photoactivation enabled the phospho-peptides to target 14-3-3 proteins and displace endogenous ligands from 14-3-3 binding. Due to the loss of 14-3-3 function, the real-time observation of aberrant cell cycle progression, including G1 cell release from interphase arrest and DNA damage-induced loss of S-phase checkpoint, could be easily achieved. Similarly, these photoactive probes could also be applied to study other phosphopeptide-binding domains in a temporally controlled manner. For example, the caged phosphotyrosine sequence was developed recently for analysis of leading edge migration of tumor cells through binding to focal adhesion kinase (FAK) effectors.⁵¹

Kron and co-workers introduced the arrest of MATs cells in the G1 phase of division using a set of photocleavable yeast peptide pheromone α -factor (WHWLQLKPGQPMY).⁵² This cell cycle arrest can be used for investigating signaling events involved in the pathway and other processes related to morphogenesis and transcription in cell biology.

4.3 Photoregulation of nuclear transport and mitochondria membrane potential

In eukaryotic cells, the nuclear envelope separates the nucleus and cytoplasm where different biological functions such as DNA transcription and protein translation occur, respectively. In order to ensure the proper regulation of signaling and

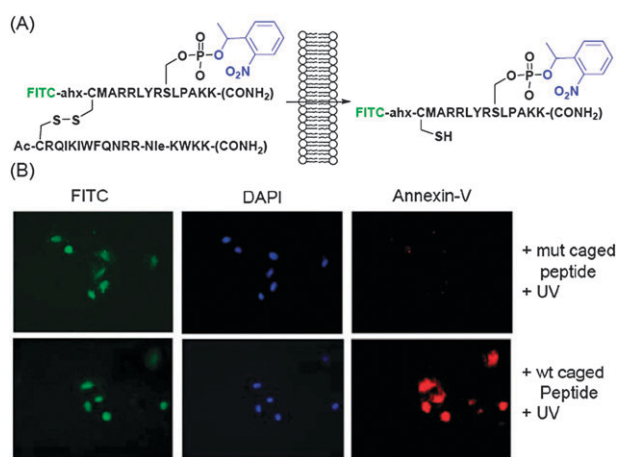


Fig. 16 (A) Caged phosphopeptides. (B) U2OS cells treated with mutant or wild-type caged peptides for analysis of programmed cell death 9 h after UV irradiation. Adapted with permission from ref. 50. Copyright 2004, Nature Publishing Group.

metabolism of a living cell, nuclear transport is a fundamental necessity to enable molecular exchange between the nucleus and cytoplasm. Development of sensitive and photocontrollable probes offers the possibility to investigate the intrinsic mechanism and dynamics of nuclear transport in living cells.

Sase and co-workers presented a novel photoactive molecule for real-time observation of cytoplasm-nucleus transport in living cells by using a caged nuclear localization signal (NLS), one classical basic peptide sequence to recognize the import receptor proteins.⁵³ The lysine-128 residue in SV40 T-antigen NLS was converted to a caged lysine with recognition blocked by a photocleavable 4,5-dimethoxy-2-nitrobenzyl carbonyl molecule. The caged NLS was then conjugated to Cy7-labelled BSA protein and microinjected into HeLa cells. Brief UV illumination activated NLS recognition sequence and led to the spatiotemporal translocation of fluorescent BSA. Related approach based on the photocaged proteins was also applied to real-time regulation of the nuclear export in living cells as described previously.¹⁷

Mitochondria play an important role in cellular biology by generating adenosine triphosphate (ATP) and regulating cellular metabolism. For studying mitochondrial functions, protonophores containing conjugated π -bond systems are commonly used to dissipate electrical potential ($\Delta\Psi_m$) which exists across the inner mitochondrial membrane and drives ATP production and Ca^{2+} uptake. These protonophores are lipophilic and membrane permeant, making them difficult to apply in specific predefined regions. Conway and co-workers synthesized two caged mitochondrial uncouplers based on tyrothostin AG10 and studied the collapse of mitochondrial membrane potential ($\Delta\Psi_m$) consistent with the photorelease of AG10 (Fig. 17).⁵⁴ The localized $\Delta\Psi_m$ depolarisation, observed from the loss of fluorescence from cell loaded $\Delta\Psi_m$ -sensitive dye, was not prevented by antioxidants, indicating the distinction from light-induced photodynamic damage. The photoreleased AG10 was incorporated into nearby intracellular membranes but not equilibrated throughout the cell, which was useful to investigate the mechanism of localized influence on mitochondrial function.

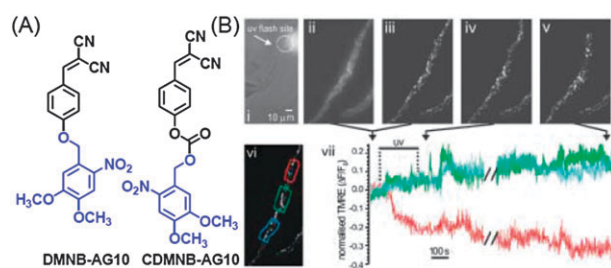


Fig. 17 (A) Structure of caged AG10; (B) localised mitochondrial depolarisation following intracellular photolytic release of AG10. Copyright 2009, Royal Society of Chemistry.

4.4 Photoactive molecules in signal transduction

Signal transduction indicates the processes by which a cell converts one kind of signal or stimulus into another, usually involving ordered sequences of biochemical reactions inside the cell. Here we will mainly introduce the caged signaling molecules such as neurotransmitters and second messengers to obtain photoregulation of signal transduction in cell biology.

4.4.1 Photoactive neurotransmitters. Caged neurotransmitters, one of the most successful photoactive probes, have been applied to investigate the kinetics and mechanistic aspects in neural sciences. Neurological process involves the response of neurons to stimuli and the transmission of stimuli to the central nervous system, which processes the information and sends responses to other parts of the system. By stimulating neurons with light, investigations into neuronal functions could be obtained which benefited greatly from the advantages of photorelease technology due to its fast delivery, specifically designed resolution and minimal cellular damage. Photoactivation of caged neurotransmitters has revealed great value in manipulating neuronal activity.^{1,2,5} Among them, caged glutamate has been widely used for conferring light sensitivity to neurons because this transmitter can excite most mammalian neurons.⁵⁵ Wieboldt and co-workers developed carboxyl-2-nitrobenzyl (CNB) glutamate derivatives with desirable properties suitable for kinetic study of glutamate receptors.⁵⁶ Laser photolysis of caged glutamate triggered the local release of free glutamate, resulting in the generation of action potentials in neurons at the release site. This method, combined with whole-cell voltage-clamp recordings, has been successfully used to investigate the locations of neurons providing excitatory input to layer 4B neurons in the primary visual cortex of macaque monkey.⁵⁷ Additionally, Matsuzaki *et al.* utilized two-photon uncaging of 4-methoxy-7-nitroindoline (MNI) caged glutamate to systematically investigate glutamate receptor functions at the level of individual synapse (Fig. 18). The hippocampal slice preparations demonstrated the distribution of functional AMPA-sensitive glutamate receptors was highly dependent on spine geometry, which supplied the possibility for the spatiotemporal regulation of glutamatergic synaptic transmission in the brain.⁵⁸ Similarly, Chiu *et al.* offered a new light-addressable nanocapsules as physical cages for spatiotemporally delivering precise signals to single cells which has been transfected to express the M1 acetylcholine receptor and has been loaded with the calcium indicator.^{59,60} This nanocapsules based physical cages exhibited a strong two-photon

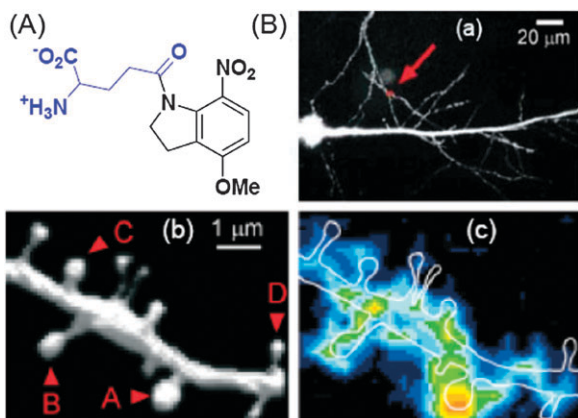


Fig. 18 (A) Structure of MNI-glutamate; (B) functional mapping of glutamate sensitivities in hippocampal neurons. (a) Fluorescence image of a CA1 pyramidal neuron. (b) 3-D reconstruction of fluorescent images. (c) Maximal intensity plot of the glutamate sensitivity for the four sections. Copyright 2001, Nature Publishing Group.

absorption cross section and rapid uncaging process. Two-photon photolysis of nanocapsules resulted in the cellular response and increasing of the intracellular calcium.

Another important transmitter is GABA (γ -aminobutyrate), the chief inhibitory neurotransmitter in the mammalian central nervous system. CNB-caged GABA is commercially available and is one of the most widely used transmitters in brain slice experiments. However, this molecule is susceptible to hydrolysis and has been reported to inhibit GABA-mediated synaptic currents at low concentrations. Ogden and co-workers recently found laser photolysis of DPNI-caged GABA at low concentration could be applied to investigate the properties and distribution of GABA receptors with high spatial resolution in hippocampal neurons.⁶¹

4.4.2 Photoactive molecules in second messenger systems.

Binding of a neurotransmitter to a membrane-spanning receptor protein creates a signal that diffuses within the cell and triggers cell response. This signal, called second messenger, can be synthesized or secreted in specific reactions by enzymes or ion channels. Photocontrolled release of the signal, including calcium ion, nitric oxide, cyclic nucleotide and inositol triphosphate, provides a simple and straightforward approach to study the biological or physiological response towards the increase of second messenger at the single-cell level.^{1,2,5}

The intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) acts as an important second messenger and is responsible for regulating diverse functions in many cell types. The strategy for Ca^{2+} release is normally based on the affinity toward the chelator. Photoactivation of caged chelator leads to structural rearrangement and significantly decreases the calcium affinity. Since the first caged Ca^{2+} chelates, nitr-series, developed by Tsien's group in 1986, different types of caged Ca^{2+} effectors have been extensively exploited to study many biological processes including signal transduction, neurotransmitter secretion, muscle contraction and ion channel regulation.⁶²

Li and co-workers recently developed a new imaging technique named LAMP (local activation of molecular fluorescent

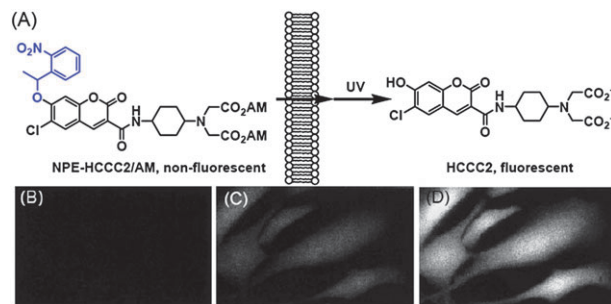


Fig. 19 (A) Structure and activation mechanism of an imaging assay of gap junctional communication. (B-D) Fluorescence imaging of primary human fibroblasts prior to (B), after 0.2 s (C) and 0.6 s (D) UV illumination. Adapted with permission from ref. 63. Copyright 2005, Nature Publishing Group.

probes) for measuring molecular transfer rates across gap junction connexin channels, by virtue of NPE-HCCC2/AM, one caged coumarin fluorophore with high uncaging cross section. This technique, involving local activation of fluorescent molecular probes, was applied to study the regulation of gap junction coupling by intracellular Ca^{2+} with high spatio-temporal resolution.⁶³ They loaded human fibroblasts with NPE-HCCC2/AM and a long wavelength fluorescent Ca^{2+} indicator, whose individual fluorescent signals were imaged concurrently (Fig. 19). With this dual-color imaging technology, the authors successfully identified a cellular signaling pathway that strongly inhibited cell coupling. Moreover, they also improved this method to non-invasively study cell coupling in living worms by using a bioconjugate of caged dye, dextran-CANPE-HCC, with prolonged cellular retention time.⁶⁴

In addition to the photocage technology, another approach for Ca^{2+} binding and transporting based on photoinduced electron transfer was reported. Gust and co-workers introduced a redox-sensitive quinone-based Ca^{2+} -binding complex which diffused through the membrane. Illumination of a photoactive molecule embedded in membrane formed a radical cation, which oxidized the quinone complex and broke the calcium binding.⁶⁵ Ca^{2+} was released at the inner membrane and continued to transport because the quinone complex was regenerated.

Nitric oxide (NO) is another important messenger molecule involved in many physiological processes such as neurotransmission, vasodilatation and hormone secretion. Different types of NO donors which are sensitive to pH, temperature, light or enzymes have been synthesized and used in biological applications to control the release of NO at a selected site.⁶⁶ Guillemette and co-workers demonstrated the strategy to control the activity of nitric oxide synthase (NOS) through the activation of NOS inhibitor from a photosensitive prodrug, Bhc-1400 W, which restored the inhibitory molecule 1400 W upon illumination.⁶⁷ Recently, the group of Sortino introduced NO donor containing a DNA intercalator binder.⁶⁸ This conjugate exhibited high binding affinity towards DNA and generated NO in the close proximity with controlled visible light irradiation, offering the great advantages for bio-applications (Fig. 20).

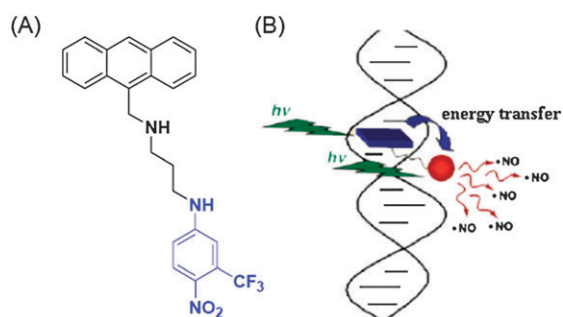


Fig. 20 (A) Structure of NO donor; (B) activation mechanism of photorelease of NO in DNA proximity. Copyright 2008, Royal Society of Chemistry.

Cyclic nucleotides such as adenosine 3',5'-cyclic monophosphate (cAMP) and cyclic guanine monophosphate (cGMP) are important in many biological processes for intracellular signal transduction. Caged cAMP or cGMP derivatives have been developed and used to analyze biological responses, such as inward currents and synaptic regulation of afterpotentials, to cellular increase in cyclic nucleotide levels.¹ Recently, Harz and co-workers investigated the spatiotemporal effects of cAMP gradients to the turning behavior of neuronal growth cones by photorelease of caged cAMP.⁶⁹ They generated an intracellular cAMP concentration gradient through locally irradiating caged cAMP at one side of growth cones of chick sensory neurons, and showed only certain patterns of cAMP release could induce growth cone turning. In another report, Jalink *et al.* utilized FRET-based sensors to study gap junction mediated cAMP transfer in Rat-1 cells quantitatively.⁷⁰ Photorelease of caged cAMP resulted in the selective increase of intracellular cAMP level, which was transferred to adjacent cells within seconds in a connexin 43-dependent manner.

The photoactive technology has been successfully employed to investigate the physiological processes and intrinsic dynamic properties of cell biology. In these studies, the most widely used photoactive molecules are based on 2-nitrobenzyl (NB) or its derivatives, which suffers from slow release rate and formation of reactive aldehyde side products upon photolysis.^{1-4,62} In addition, specific cellular target and efficient membrane penetration are also challenges for their applications in living systems. Extensive efforts therefore need to be further devoted to address these concerns by developing improved photoactive caging molecules and strategies.

Conclusions

This review described the latest developments in photoactive technology with an emphasis on their applications in molecular imaging and cell biology. This technology utilizes light to activate photosensitive molecules, allowing precise regulation of biological processes in a time and site-specific resolution, and has been proven highly beneficial for studying biological systems by providing a rather simple and direct method. Despite the great promises of photoactive technology in cell biology, a lot of demanding biological problems certainly remain to be fully elucidated and substantial efforts still need to be conducted to solve these problems based on this

extraordinary technology. One major challenge of the photoactive technology is to develop new types of photoactive molecules with higher stability and photochemical properties, which will enable the site-specific modification of biomolecules and selective recognition of specific targets in living systems. Development of sophisticated analytical laser equipment and the design of new photoactive moieties with higher two-photon absorption cross section are another rapidly growing field to allow non-invasive photolysis with minimum cellular photodamage and real-time visualization of biological processes ranging from single cells to multicellular organisms. Additionally, the future perspective of photoactive technology also requires interdisciplinary cooperation not only to achieve better understanding of biological systems and address many new biological questions, but also to discover new targets and novel principles in cell biology. Currently, the advances of this technique are just recognized at its infancy stage. With all the innovations in chemistry, biology and photophysics, photoactive technique will continue to expand and provide more exciting new insights in both basic biological studies and clinical practice.

Acknowledgements

The authors gratefully acknowledge the start-up, URC (RG56/06) and A*Star BMRC (07/1/22/19/534) grants in Nanyang Technological University, Singapore.

Notes and references

- 1 G. Mayer and A. Heckel, *Angew. Chem., Int. Ed.*, 2006, **45**, 4900.
- 2 H. M. Lee, D. R. Larson and D. S. Lawrence, *ACS Chem. Biol.*, 2009, **4**, 409.
- 3 G. C. R. Ellis-Davies, *Nat. Methods*, 2007, **4**, 619.
- 4 A. P. Pelliccioli and J. Wirz, *Photochem. Photobiol. Sci.*, 2002, **1**, 441.
- 5 D. D. Young and A. Deiters, *Org. Biomol. Chem.*, 2007, **5**, 999.
- 6 A. Kocer, M. Walko, W. Meijberg and B. L. Feringa, *Science*, 2005, **309**, 755.
- 7 M. Volgraf, P. Gorostiza, R. Numano, R. H. Kramer, E. Y. Isacoff and D. Trauner, *Nat. Chem. Biol.*, 2006, **2**, 47.
- 8 C. Y. Chang, T. Fernandez, R. Panchal and H. Bayley, *J. Am. Chem. Soc.*, 1998, **120**, 7661.
- 9 K. Y. Zou, S. Cheley, R. S. Givens and H. Bayley, *J. Am. Chem. Soc.*, 2002, **124**, 8220.
- 10 C. Orange, A. Specht, D. Puliti, E. Sakr, T. Furuta, B. Winsor and M. Goeldner, *Chem. Commun.*, 2008, 1217.
- 11 S. Banala, A. Arnold and K. Johnsson, *ChemBioChem*, 2008, **9**, 38.
- 12 B. J. Wilkins, X. Yang and T. A. Cropp, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 4296.
- 13 Q. Shao, T. T. Jiang, G. Ren, Z. Cheng and B. G. Xing, *Chem. Commun.*, 2009, 4028.
- 14 M. E. Hahn and T. W. Muir, *Angew. Chem., Int. Ed.*, 2004, **43**, 5800.
- 15 J. P. Pellois and T. W. Muir, *Angew. Chem., Int. Ed.*, 2005, **44**, 5713.
- 16 N. Wu, A. Deiters, T. A. Cropp, D. King and P. G. Schultz, *J. Am. Chem. Soc.*, 2004, **126**, 14306.
- 17 E. A. Lemke, D. Summerer, B. H. Geierstanger, S. M. Brittain and P. G. Schultz, *Nat. Chem. Biol.*, 2007, **3**, 769.
- 18 P. R. Chen, D. Groff, J. Guo, W. Ou, S. Cellitti, B. H. Geierstanger and P. G. Schultz, *Angew. Chem., Int. Ed.*, 2009, **48**, 4052.
- 19 C. J. Chou, D. D. Young and A. Deiters, *Angew. Chem., Int. Ed.*, 2009, **48**, 5950.
- 20 W. F. Edwards, D. D. Young and A. Deiters, *ACS Chem. Biol.*, 2009, **4**, 441.

- 21 M. Endo, K. Nakayama, Y. Kaida and T. Majima, *Angew. Chem., Int. Ed.*, 2004, **43**, 5643.
- 22 H. Li, J. M. Hah and D. S. Lawrence, *J. Am. Chem. Soc.*, 2008, **130**, 10474.
- 23 S. E. Cellitti, D. H. Jones, L. Lagpacan, X. S. Hao, Q. Zhang, H. Y. Hu, S. M. Brittain, A. Brinker, J. Caldwell, B. Bursulaya, G. Spraggon, A. Brock, Y. Ryu, T. Uno, P. G. Schultz and B. H. Geierstanger, *J. Am. Chem. Soc.*, 2008, **130**, 9268.
- 24 M. R. Banghart, A. Mourot, D. L. Fortin, J. Z. Yao, R. H. Kramer and D. Trauner, *Angew. Chem., Int. Ed.*, 2009, **48**, 9097.
- 25 S. Loudwig and H. Bayley, *J. Am. Chem. Soc.*, 2006, **128**, 12404.
- 26 X. J. Tang and I. J. Dmochowski, *Mol. BioSyst.*, 2007, **3**, 100.
- 27 W. T. Monroe, M. M. McQuain, M. S. Chang, J. S. Alexander and F. R. Haselton, *J. Biol. Chem.*, 1999, **274**, 20895.
- 28 L. Krock and A. Heckel, *Angew. Chem., Int. Ed.*, 2005, **44**, 471.
- 29 D. D. Young, J. M. Govan, M. O. Lively and A. Deiters, *ChemBioChem*, 2009, **10**, 1612.
- 30 A. Yamazawa, X. G. Liang, H. Asanuma and M. Komiyama, *Angew. Chem., Int. Ed.*, 2000, **39**, 2356.
- 31 Y. M. Kim, J. A. Phillips, H. P. Liu, H. Z. Kang and W. H. Tan, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 6489.
- 32 S. G. Chaulk and A. M. MacMillan, *Nucleic Acids Res.*, 1998, **26**, 3173.
- 33 S. G. Chaulk and A. M. MacMillan, *Nat. Protoc.*, 2007, **5**, 1052.
- 34 S. Shah, S. Rangarajan and S. H. Friedman, *Angew. Chem., Int. Ed.*, 2005, **44**, 1328.
- 35 S. Shah, P. Jain, A. Kala, D. Karunakaran and S. H. Friedman, *Nucleic Acids Res.*, 2009, **37**, 4508.
- 36 X. J. Tang and I. J. Dmochowski, *Angew. Chem., Int. Ed.*, 2006, **45**, 3523.
- 37 D. D. Young, H. Lusic, M. O. Lively, J. A. Yoder and A. Deiters, *ChemBioChem*, 2008, **9**, 2937.
- 38 D. Matsunaga, H. Asanuma and M. Komiyama, *J. Am. Chem. Soc.*, 2004, **126**, 11452.
- 39 H. Ando, T. Furuta, R. Y. Tsien and H. Okamoto, *Nat. Genet.*, 2001, **28**, 317.
- 40 I. A. Shestopalov, S. Sinha and J. K. Chen, *Nat. Chem. Biol.*, 2007, **3**, 650.
- 41 X. J. Tang, S. Maegawa, E. S. Weinberg and I. J. Dmochowski, *J. Am. Chem. Soc.*, 2007, **129**, 11000.
- 42 S. B. Cambridge, D. Geissler, F. Calegari, K. Anastassiadis, M. T. Hasan, A. F. Stewart, W. B. Huttner, V. Hagen and T. Bonhoeffer, *Nat. Methods*, 2009, **6**, 527.
- 43 J. A. Theriot and T. J. Mitchison, *Nature*, 1991, **352**, 126.
- 44 P. Roy, Z. Rajfur, D. Jones, G. Marriott, L. Loew and K. Jacobson, *J. Cell Biol.*, 2001, **153**, 1035.
- 45 M. Ghosh, X. Y. Song, G. Mouneimne, M. Sidani, D. S. Laurence and J. S. Condeelis, *Science*, 2004, **304**, 743.
- 46 S. Petersen, J. M. Alonso, A. Specht, P. Duodu, M. Goeldner and A. del Campo, *Angew. Chem., Int. Ed.*, 2008, **47**, 3192.
- 47 A. M. Kloxin, A. M. Kasko, C. N. Salinas and K. S. Anseth, *Science*, 2009, **324**, 59.
- 48 D. Liu, Y. Xie, H. Shao and X. Jiang, *Angew. Chem., Int. Ed.*, 2009, **48**, 4406; J. Auernheimer, C. Dahmen, U. Hersel, A. Bausch and H. Kessler, *J. Am. Chem. Soc.*, 2005, **127**, 16107.
- 49 J. Nakanishi, Y. Kikuchi, S. Inoue, K. Yamaguchi, T. Takarada and M. Maeda, *J. Am. Chem. Soc.*, 2007, **129**, 6694.
- 50 A. Nguyen, D. M. Rothman, J. Stehn, B. Imperiali and M. B. Yaffe, *Nat. Biotechnol.*, 2004, **22**, 993.
- 51 D. Humphrey, Z. Rajfur, M. E. Vazquez, D. Scheswohl, M. D. Schaller, K. Jacobson and B. Imperiali, *J. Am. Chem. Soc.*, 2005, **280**, 22091.
- 52 L. L. Parker, J. W. Kurutz, S. B. H. Kent and S. J. Kron, *Angew. Chem., Int. Ed.*, 2006, **45**, 6322.
- 53 Y. Watai, I. Sase, H. Shiono and Y. Nakano, *FEBS Lett.*, 2001, **488**, 39.
- 54 N. Avlonitis, S. Chalmers, C. McDougall, M. N. Stanton-Humphreys, C. T. A. Brown, J. G. McCarron and S. J. Conway, *Mol. BioSyst.*, 2009, **5**, 450.
- 55 E. M. Callaway and R. Yuste, *Curr. Opin. Neurobiol.*, 2002, **12**, 587.
- 56 R. Wieboldt, K. R. Gee, L. Niu, D. Ramesh, B. K. Carpenter and G. P. Hess, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 8752.
- 57 N. H. Yabuta, A. Sawatari and E. M. Callaway, *Science*, 2001, **292**, 297.
- 58 M. Matsuzaki, G. C. R. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Lino and H. Kasai, *Nat. Neurosci.*, 2001, **4**, 1086.
- 59 K. A. Dendramis, P. B. Allen, P. J. Reid and D. T. Chiu, *Chem. Commun.*, 2008, 4795.
- 60 K. A. Dendramis and D. T. Chiu, *J. Am. Chem. Soc.*, 2009, **131**, 16771.
- 61 F. F. Trigo, G. Papageorgiou, J. E. T. Corrie and D. Ogden, *J. Neurosci. Methods*, 2009, **181**, 159.
- 62 G. C. R. Ellis-Davies, *Chem. Rev.*, 2008, **108**, 1603.
- 63 K. Dakin, Y. R. Zhao and W. H. Li, *Nat. Methods*, 2005, **2**, 55.
- 64 Y. M. Guo, S. W. Chen, P. Shetty, G. H. Zheng, R. L. Lin and W. H. Li, *Nat. Methods*, 2008, **5**, 835.
- 65 I. M. Bennett, H. M. V. Farfano, F. Bogani, A. Primak, P. A. Liddell, L. Otero, L. Sereno, J. J. Silber, A. L. Moore, T. A. Moore and D. Gust, *Nature*, 2002, **420**, 398.
- 66 P. G. Wang, M. Xian, X. P. Tang, X. J. Wu, Z. Wen, T. W. Cai and A. J. Janczuk, *Chem. Rev.*, 2002, **102**, 1091.
- 67 H. J. Montgomery, B. Perdicakis, D. Fishlock, G. A. Lajoie, E. Jervis and J. G. Guillemette, *Bioorg. Med. Chem.*, 2002, **10**, 1919.
- 68 F. L. Callari and S. Sortino, *Chem. Commun.*, 2008, 1971.
- 69 S. Munck, P. Bedner, T. Bottaro and H. Harz, *Eur. J. Neurosci.*, 2004, **19**, 791.
- 70 B. Ponsioen, L. van Zeijl, W. H. Moolenaar and K. Jalink, *Exp. Cell Res.*, 2007, **313**, 415.