Regulating gene expression with light-activated oligonucleotides

XinJing Tang and Ivan J. Dmochowski*

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Since the development of light-responsive amino acids, the activity of numerous biomolecules has been photomodulated in biochemical, biophysical, and cellular assays. Biological problems of even greater complexity motivate the development of quantitative methods for controlling gene activity with high spatial and temporal resolution, using light as an external trigger. Photoresponsive DNA and RNA oligonucleotides would optimally serve this purpose, but have proven difficult to expand from proofs-of-concept to in vivo experiments. Until recently, the development of this technology was limited by the synthesis of oligonucleotides whose function could be significantly modulated with near-UV light. New synthetic protocols and strategies for both up- and down-regulating gene activity finally make it possible to address biological considerations. In the near future, we can expect photoresponsive DNA and RNA molecules that are relatively non-toxic, nuclease-resistant, and maintain their specificity and activity in vivo. Quantitative, laser-initiated methods for controlling DNA and RNA function will illuminate new areas in cell and developmental biology.

1. Introduction

In nature, relatively simple photochemical processes often accomplish complex biological tasks. For example, the visual process by which we are reading this article is initiated in the retina by the 7-transmembrane protein, rhodopsin.1–3 This G-protein-coupled receptor (GPCR) adopts an inactive form when covalently bound to 11-cis-retinal, which prevents binding to peripheral membrane proteins, including the heterotrimeric G protein transducin. In this “caged” state, rhodopsin is transiently blocked from performing its biological function. Absorption of a photon induces the rapid (200 fs) isomerization of 11-cis-retinylidene to the all-trans-retinylidene, and triggers a conformational change in rhodopsin that activates transducin. The subsequent
visual signaling cascade causes cGMP-gated ion channels to close, which leads to a hyperpolarized membrane potential. Multiple photocycles generate an electric impulse that is transduced to the brain via the basal ganglia and optic nerve. Remarkably, this entire process is initiated by a single photon! In 1943 Karrer et al. synthesized the first azobenzene-modified amino acids. Nitrobenzoylcarbonylglycine was later introduced by Barltop and Schofield in 1962, the same year that Watson, Crick, and Wilkins jointly received the Nobel Prize for determining the structure of DNA. Since that time, chemists have succeeded in caging myriad biomolecules using “light switches” with similar functionality to 11-cis-retinal. This review highlights key examples of caged biomolecules, while focusing on the development of probes for controlling the structure and function of oligonucleotides. One frontier application involves photoregulating the location, timing, and quantitative level of protein expression in living biological specimens.

The visual signaling cascade illustrates several important features of photoactive chromophores for controlling biological processes. First, photochemical processes are typically very efficient, where an absorbing molecule can be excited with just one or two photons. In rhodopsin, high signal-to-noise is achieved based on the stability of 11-cis-retinal in the dark, the rapid and high-quantum-yield cis → trans photoconversion, and the differential activity of rhodopsin bound to cis- and trans-retinal. This makes it possible in low light conditions to detect signals arising from activated rhodopsin in just a few rod cells. Electrical impulses originating from these cells are transmitted over many centimetres. Remarkably, our eyes achieve 15–20 ms temporal resolution, which, coincidentally, allows us to perceive flicker on a computer monitor in ambient light if the refresh rate is set below 60 Hz. Researchers routinely apply caging strategies for photoinitiating biological processes that occur on nanosecond–millisecond timescales.

Photochemical processes can also be photoinitiated with high spatial resolution. The Rayleigh criterion for resolving the diffraction patterns from two point sources in an image plane is given by the radius of the Airy disk, \( r_{\text{Airy}} = 0.61 \times \lambda/\Delta x \), where \( \lambda \) is the wavelength of the light source and \( \Delta x \) is the numerical aperture of the objective lens that focuses the beam. In our visual system, the cornea at the front of the eyeball works in tandem with a double convex crystalline lens to focus visible light onto the back of the retina. This spatially constrains the photochemical process within specific rod and cone cells, and provides nearly diffraction-limited (~0.5 micron) visual acuity under ideal conditions. Focused lasers in various configurations, including widefield, confocal, and two-photon microscopes as well as whole-animal optical scanners, can initiate the photochemical reaction in biological regions of interest on widely varying size scales (centimetres to sub-micron). Most recently, Betzig et al. demonstrated that it is possible to achieve nanometre spatial resolution while imaging photoactivatable (\( \lambda = 405 \text{ nm} \)) fluorescent protein molecules in fixed whole cells.

A third important feature of photochemical processes is that visible photons are relatively benign and yet can penetrate centimetres into living tissue, as seen by transilluminating your hand with a flashlight. For triggering biochemical events, it is also important that light is orthogonal to other stimuli that typically elicit biological responses. This ensures, for example, that our visual cortex does not suddenly record false images if the ambient temperature increases!

Based on these advantages, caging/photostimulation strategies are being applied with increasing regularity and sophistication in cellular, neuro-, and developmental biology, as well as biochemical and biophysical studies. Researchers have designed photoactive neurotransmitters, amino acids, peptides, proteins, hormones, and fluorophores. Several texts, including Mayer and Heckel’s recent review, detail the biological activity of these caged biomolecules and the organic mechanisms of their photostimulation. The chemistry of the 2-nitrobenzyl moiety and derivatives has been most widely investigated. Other useful protecting groups include \( \alpha \)-alkylated aryl ketones, benzoins, \( \beta \)-hydroxyphenacyl, coumarins, nitroindolines, and related chromophores. Many photoprotecting groups, as well as caged biomolecules, are now commercially available.

This review relates broadly to metal-mediated DNA photodecay, non-specific UV-mediated DNA damage, and the widespread use of photoprotecting groups in oligonucleotide synthesis, as exemplified by Pitsch and coworkers’ solid-phase RNA synthesis and work by Fodor et al. using spatially resolved, sequential photodeprotecting steps, which led to the realization of the Affymetrix GeneChip. A variety of photochemical methods have been devised for ligating two oligonucleotides, for example, via anthracene cyclodimers or adducts of 5-vinyldeoxyuridine. Also relevant are previous studies with DNA oligonucleotides that contain a photoactive brominated or iodinated nucleoside. Most common are 5-bromo-2′-deoxyuridine (BrdU), 8-bromo-2′-deoxyadenosine, 5-bromo-2′-deoxycytidine, and 8-bromo-2′-deoxyguanosine. These photoactive nucleosides are readily incorporated into oligonucleotides by solid-phase synthesis and facilitate UV-crosslinking experiments with DNA-binding proteins. Similarly, diazirines as well as commercial 6-thio-dG, 4-thio-dT, and 4-thio-dU are useful...
modifications for photocrosslinking and photoaffinity labelling experiments. Photocatalytic dehalogenation of 5-halouracil-containing DNA and subsequent abstraction of hydrogen from a nearby ribose can also probe different DNA structures. However, generation of uracil-5-yl radicals with 302 nm excitation, although atom-specific when performed in vitro, is likely to be incompatible with living cells, due to the non-specific absorbance at this wavelength and potential for UV damage. This review focuses on methods for controlling the structure and function of oligonucleotides, where the regulation of in vivo gene expression should be performed at wavelengths greater than 350 nm.

2. General methods for regulating gene expression

Many strategies for up- and down-regulating gene expression have already been developed for studies in cell culture and a variety of model organisms, including chickens, mice, fish, frogs, sea urchins, flies and worms. Gene function is typically determined by perturbing the expression of one or more genes, and observing the biochemical or phenotypic response. However, many genes are co-opted to perform multiple functions, at different times and in different tissues.

One example in the developing zebrafish (Danio rerio) embryo is chordin, which is important in dorsal-ventral specification at 4-6 hours post fertilization (hpf) and later in the developing brain at 12 hpf. Thus, methods for studying chordin’s function in the brain at 12 hpf would ideally leave chordin untouched throughout the embryo until the appointed time. Furthermore, the complete knockdown of genes using morpholino- or peptide nucleic acid (PNA)-based antisense molecules can be embryonically lethal, based on the requirement for a gene’s expression in early development. The death of the embryo masks the function(s) of the down-regulated protein. These experimental challenges motivate the development of versatile and delicate photochemical probes of gene function in space and time.

Researchers are also still limited in their ability to answer questions regarding the in vivo concentration of proteins, acting singly or in tandem with other partners. Many signaling molecules, such as fibroblast growth factor (fgf) or squint, have been shown to diffuse large distances and function in a concentration-dependent manner. Cells producing these signaling molecules can serve as beacons to guide the movements and gene expression patterns of other nearby cells. Conventional molecular approaches to manipulating genes in live embryos or animals, e.g. stable transgenics, transient transgenics by introducing DNA, mRNA, or antisense molecules, or diffusible small molecules, do not allow the modulation of gene expression on a cell-by-cell basis. Microsurgeries to implant protein-labeled beads or transplant foreign, protein-secreting cells into the organism create new opportunities. However, such surgeries are challenging, and in each case it can be difficult to separate the phenotypic response from the unintended consequences of the wound. The use of heat-responsive promoters, which can be either activated in individual cells with a laser or more broadly by warming an entire organism, circumvents some of these problems. However, many organisms have strict temperature requirements, and temperatures can vary widely over very short distances, even nanometres, which makes this approach only semi-quantitative. Thus, even basic questions, such as how cells within a living organism communicate over large (μm-mm) distances, require the development of more sophisticated techniques for controlling the concentration, localization, and timing of protein expression.

To address important questions involving gene expression, caged small molecules and proteins have been developed, each with some particular advantages. For example, Koh and coworkers’ caged tamoxifen derivatives can be readily diffused into cells and selectively antagonize transcription at estrogen response elements when exposed to UV light. The spatial and temporal resolution that can be achieved by this approach, as well as its general applicability to regulating gene expression in biological specimens other than cells, remain to be investigated. Complementary reverse chemical genetics approaches have focused on screening large, diverse libraries of organic molecules in order to identify small molecule “drugs” that generate a desired phenotype and are specific for a given target. However, finding such molecules is expensive and time-consuming, and after the observation of a new phenotype, it can be challenging to identify the biomolecular target(s). By comparison, caged oligonucleotides represent a forward chemical genetics approach that makes use of available genomic and biochemical information to target specific protein-coding DNA and mRNA.

Caged transcription factors, such as GAL4VP16, can be applied to any system containing a protein-coding plasmid that includes the necessary upstream activation site (UAS). However, the difficult synthesis, purification, and cellular introduction of these caged proteins limit their utility for many experiments. GAL4VP16 that is microinjected into Xenopus embryos does not diffuse far from the site of injection, whereas mRNA typically distributes uniformly. Blaettler and coworkers’ caged protein toxins were shown to diffuse readily into cells, and interfere with ribosomal protein expression, but it remains a challenge to control their concentration or localization within the cell. In order to circumvent these problems, Schultz et al. have developed methods for genetically encoding caged proteins that contain o-nitrobenzylated amino acids. By this method, it is possible to incorporate one or more caging moieties site-specifically within the protein. However, the molecular biology expertise that is required to develop orthogonal tRNA-synthetase pairs from E. coli and incorporate these within a highly engineered strain of S. cerevisiae, is not yet accessible to many groups, and this approach is limited currently to studies in yeast. A fundamental problem with caged proteins is that the protecting group must be redesigned and optimized for each new target by varying, for example, the position of the caged amino acid. In contrast, a general method of controlling DNA and RNA function with light would make myriad proteins and signaling pathways immediately accessible for study.

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Caged oligos can be tailored to meet most experimental requirements. Oligonucleotides are delivered intracellularly by many different methods, including passive diffusion, electroporation, transport mediated by lipid, cationic, or viral carriers, and microinjection. Cell stability can be enhanced considerably by modifying the backbone of the oligonucleotide, as exemplified by phosphorothioate,69 peptide,61 morpholine,59 and ‘locked’70 nucleic acids. Light-activated methods for controlling the binding of antisense oligodeoxynucleotides (asODNs) to target mRNA would permit the reduction of protein expression in a cell- or tissue-specific fashion, by either sterically blocking the ribosome59,60 or recruiting ribonucleases71–73 that bind specifically to DNA/RNA duplexes. asODNs have proven effective in gene silencing in many experimental systems,74–76 and are being evaluated as treatments for cancer and other diseases in human clinical trials.76–79 Thus, it appears likely that methods for controlling oligonucleotide structure and function will soon achieve biomedical significance.77 In order to consider the possible applications of this technology, we must first review the current status of photoactive oligonucleotides.

3. Synthesis of light-responsive oligonucleotides

“Caged DNA” is produced in nature with profound biological consequences that include skin cancer, cataracts, and other common disorders.82 Ultraviolet light damages DNA by catalysing covalent bond formation between adjacent pyrimidines, which generates cis-syn cyclobutane pyrimidine dimers (CPDs) as the most common lesion.80 CPDs block DNA replication by high-fidelity DNA polymerases. The CPDs formed in the most significant quantity are the cis-syn cyclobutane dimer of two thymine bases and the corresponding 6-4 photoproduct (Fig. 2). Lewis and Hanawalt first showed that irradiation with >290 nm light caused the ligation of multiple dT10 strands, templated by poly(dA), and that subsequent irradiation with 254 nm light resulted in cleavage of the UV-induced linkages.81 Based on these results, it was suggested that the pyrimidine dimer may have served as a primordial ‘ligase’ to generate short polynucleotide (caged) strands. More common are intra-strand thymine dimers, which contain the standard internucleotide phosphate and are efficiently repaired by photolyase. Thus, living cells, which are exposed to UV light from the sun, have developed methods for removing the lesions that cage their function.

Pioneering work in 1977 by Schlaeger and Engels led to the design of the first photoactive nucleotide, an adenosine cyclic 3’,5’-monophosphate (cAMP) protected as the o-nitrobenzyl ester.24 The following year, Hoffman et al. produced the first caged ATP molecule.82 Improved yields of photoactivated ATP were achieved using the 1-(2-nitro)phenylethyl (NPE) caging moiety, which led to the successful photomodulation of ATPase activity in cells. These photoactive nucleotides and subsequent versions have facilitated a wide range of biological investigations.26,27,38,83

In marking the 30th anniversary of caged cAMP, we note the pervasive adoption and adaptation of caging methodologies across biochemistry and biology. By comparison, photoactive oligonucleotides have proven more difficult to adapt to the needs of biological researchers. A recent literature search of “caged ATP” identified more than 250 original citations from 1982 to the present, whereas a much broader search of “caged”, “light-activated”, or “photoactive DNA/RNA” yielded less than 100 references, most of which appear in this review. The initial development of near UV-responsive photoactive oligonucleotides was slowed by the challenges of chemically synthesizing long-chain poly-nucleotides84 and subsequently by the incompatibility of solid-phase chemistry with delicate near-UV-responsive protecting groups. Notably, after the first introduction of caged cAMP and ATP,24,82 it took almost 15 years for the development of DNA synthesis building blocks that would allow the incorporation of near-UV photoactive moieties within oligonucleotides.85 Progress in this interdisciplinary field has relied on the synthesis, integration, and validation of new photoactive probes, photochemical and biophysical characterization of modified oligonucleotides, and myriad biochemical and cellular assays, with important contributions from many established groups. Several new investigators, including Sheppard,86–89 Belshaw,90 Silverman,91 Friedman,92 Heckel,93–97 and Dmochowski98,99 have made additional recent advances. Researchers focused initially on designing photoactive oligonucleotides as probes of DNA damage (with the introduction of abasic sites and strand breaks), as well as DNA repair mechanisms.85,100,101 It did not go unnoticed that methods for photochemically modifying DNA strands also opened possibilities for controlling DNA conformation and function, e.g., by activating prodrug forms of asODNs and ribozymes.

Urdea et al. generated what was probably the first near-UV photoactivatable oligonucleotide by synthesizing the protected abasic 1’-O-(2-nitrobenzyl)-2’-deoxyribose and incorporating this building block within a single-stranded oligonucleotide.85 It proved somewhat more difficult to phototrigger strand breaks in DNA, and early examples incorporated the relatively stable cis-syn thymine dimer100 or C4’-phenyl selenide derivative of adenosine101 into the oligodeoxynucleotide. Activation of these compounds required prolonged far-UV exposure or anaerobic conditions. Pioneering work by Taylor and Ordoukhanian in 1995 incorporated a bridging photoactive o-nitrobenzyl...
moiety within the phosphate backbone of a DNA hairpin (Fig. 3). Near UV irradiation under ambient conditions induced a strand break, thereby revealing an 18-mer oligonucleotide that exhibited a 9-fold increase in affinity for a complementary DNA sequence in M13 clone CW1. More recently, a variety of methods for generating site-specific DNA strand breaks as well as abasic sites in DNA and RNA oligonucleotides have been described.

The incorporation of photoactive protecting groups that are orthogonal to acid or base-labile moieties has led to oligonucleotides with new functionality. Famulok et al. demonstrated the utility of photoactive linkers for purifying oligonucleotides by affinity chromatography. In one example, a ribozyme was evolved for the aldol reaction, whereby a photocleavable bio- tin tag enforced the enrichment of sequences that yielded the aldol reaction product. Light-activated drug delivery strategies have also been developed by Tanabe et al. and Saito et al., in which the 5'-end of a DNA hairpin was labeled with a naphthalene quencher and the 3'-end was labeled with a photoactive o-nitrobenzyl or phenacyl photoactive chromophore attached to a drug-like molecule. Photorelease of the "drug" with UV light was shown to be 2–7 times more efficient when the quencher was spatially separated from the photoactive chromophore, which occurred when the stem-loop DNA linker hybridized to its DNA target. Methods for joining two oligonucleotide segments with an azo-benzene linker provide routes to photoisomerizable DNA structures.

In a different application, Silverman et al. synthesized all four NPE-modified RNA nucleotides and demonstrated their site-specific incorporation within RNA oligonucleotides by conventional solid-phase RNA synthesis, using Pitsch and coworkers’ 2'-O-trisopropylsilyl-oxymethyl (TOM) methodology. Although the incorporation of a single NPE moiety in the prototype 15-mer and 24-mer RNA oligonucleotides had a relatively small effect on RNA stability, the authors proposed joining two normally distant nucleotides within the RNA tertiary structure by a photochemical linker, as was previously done in proteins. This approach should facilitate RNA conformational studies on very short timescales. By incorporating a single NPE-modified guanosine into a 20 base RNA hairpin structure, Pitsch et al. were able to photoinitiate RNA refolding with three laser pulses, equilibrate one hour, and study the resulting interconversion between initial and final hairpin structures using NMR spectroscopy.

### 3.1 Controlling RNA degradation with caged RNAzymes and DNAzymes

Incorporating photoactive bonds within RNA or DNA also provides a method for modulating the catalytic degradation of complementary RNA. In 1998 Chaulk and MacMillan demonstrated the first caged RNA, as well as the first photochemical control of a ribozyme reaction, through the site-specific modification of a 2'-hydroxyl nucleophile with the o-(2-nitrobenzyl) moiety. By caging the hammerhead ribozyme this way, it was possible to photoregulate its endonuclease activity towards a complementary RNA substrate. This approach may be somewhat impractical for regulating genes in vivo, due to the requirement for introducing the caged (unstable) RNA molecule, as well as its activation at 308 nm. However, this general concept has been advanced by several groups (Fig. 4). Perrin et al. described a light-activated 8–17E deoxyribozyme (DR) by incorporating an adenosine analog in which the C8 locus was modified with a photoactive alkyl sulfide; DR activity was revealed in the far UV, \( \lambda_{\text{max}} = 279 \text{ nm} \). Notably, this strategy exploited the weak carbon–sulfur bond to generate a “traceless cage” that restored the C–H bond after cleavage. Unfortunately, the photoactivated DR proved to be an inefficient catalyst for RNA cleavage. In a related experiment,
Liu and Sen tethered two photoisomerizable azobenzene moieties to 8–17 DR, in either the substrate-binding arm or catalytic core. RNA cleavage activity varied more than 5-fold between the cis and trans conformations, with considerable dependence on the position of each azobenzene within the DR. Keiper and Yule went further by modifying the 10–23 DR with azobenzene attached via the ortho, meta, or para positions. Irradiation at 366 nm generated cis-azobenzene, which in the o-DR increased the rate of substrate RNA cleavage 9-fold over the trans o-DR. Multiple turnover was achieved in this system and cis o-DR was fairly stable for approximately one hour at 26 °C. This strategy presents exciting possibilities for photoregulating the degradation of target mRNA in biological specimens, including marine life, that can live comfortably at room temperature. The toxicity, nuclease resistance, and RNA cleavage efficiency of photoactive DNAs remain to be tested in vivo.

A final catalytic example comes from Young and Deiters, who returned to the problem of photoregulating the hammerhead ribozyme. Instead of labeling RNA with the photoactive moiety, which presented synthetic and experimental challenges, they sought to control RNA activity using a caged small-molecule effector. Theophylline-responsive ribozymes are known to cleave their own RNA intramolecularly (cis), as well as RNA substrates intermolecularly (trans). Labeling theophylline with different “traceless” photoactive moieties arrested cis and trans ribozyme activity; 365 nm irradiation revealed theophylline and thereby restored the allosteric binding interaction. Importantly, a caged theophylline was shown to penetrate the cell membranes of E. coli and Danio rerio and was non-toxic. Ongoing studies will reveal whether caged theophylline can be activated successfully to target ribozyme binding sites in living specimens.

3.2 Controlling enzyme activity with caged DNA and RNA

Caged DNA also makes it possible to photoregulate gene activity by controlling enzymes such as RNA polymerases. Towards this end, Komiyama and co-workers have generated a variety of caged DNA oligonucleotides with one or more azobenzenes. Transcription by RNA polymerase was essentially switched off by trans-azobenzene when this photoactive moiety was incorporated within the T7 promoter sequence. In a related study, SP6 RNA polymerase was effectively photomodulated by introducing two azobenzenes into the promoter region. trans-Azobenzene was presumed to intercalate within the DNA base stack and interfere with polymerase binding, whereas the nonplanar cis-azobenzene posed little impediment to the enzyme. Recent reviews highlight the utility of azobenzene for photoregulating the structure and function of alpha-helical peptides, as well as ion channels in excitable cells.

Unfortunately, reversible photomodulation of gene expression, although an attractive goal, may be impractical using azobenzene in many in vivo experiments, based on the instability of the cis isomer at physiological temperatures and in visible light. Maintaining the cis conformation requires repeated UV irradiation, which is incompatible with most living specimens. Similar issues have been encountered using other reversible phototriggers, such as stilbene or nitrospiropyran. The latter has been tethered at the 5'-end of homothymidine and thermally isomerized to colored merocyanine, which is an extended, pi-conjugated system. Photosomerization (λ > 490 nm) leads to the closed, colorless spiropyran. In order to exert more direct control over oligonucleotide structure and function, many proteins have been photomodulated using caged DNA or RNA with irreversible photochemistry (Fig. 5).

In our lab, DNA polymerase (Klenow) was transiently blocked using a photoactive DABSYL quencher that functioned in tandem with a nearby fluorophore. Photoactivation increased yields of primer extension 20-fold and led to a 50-fold increase in fluorescence that made it possible to monitor the uncaging process in real time. Heckel et al. showed that the binding affinity of a short, single-stranded DNA aptamer to thrombin could be transiently blocked by incorporating caged thymidine nucleobases. More recently, the active aptamer was converted to an inactive form through the formation of a hairpin, where the complementary DNA strand was caged with an NPE-modified cytidine. And Friedman et al. caged the function of a small interfering RNA (siRNA) targeting GFP that was presumed to function in the cell by blocking the interaction of the siRNA with the RNA-induced silencing complex (RISC). Optimal photomodulation of the GFP signal was achieved under conditions where 10.8% of the phosphate groups (~four per duplex) were modified with the 4,5-dimethoxy-2-nitrophe-nyethyl (DMNPE) blocking group. Non-site-specific, “statistical” labeling contributed to the modest 2-fold decrease in GFP signal that was observed upon photo-uncaging. These examples show that it is relatively straightforward to control oligonucleotide activity using a small number of photoprotecting groups, particularly in a homogeneously caged sample.

3.3 Controlling DNA–DNA and DNA–RNA hybridization with caged DNA

A different strategy for controlling gene expression involves photoregulating DNA–DNA or DNA–RNA hybridization. Controlling duplex formation in oligonucleotides has proven challenging, based on the large binding energy and specificity associated with this molecular recognition event. Attachment of azobenzene or spiropyran at the 5'-end of a short oligonucleotide was shown to have only a modest effect on hybridization to a complementary strand, irrespective of chromophore conformation. However, judicious placement of one or more blocking groups within the oligonucleotide provides a versatile strategy for controlling enzyme–nucleic acid interactions. Taylor and Ordoukhanian’s early approach of transiently blocking hybridization using a complementary DNA strand connected by a single o-nitrobenzyl ether proved to be remarkably efficient. More recently, Komiyama et al. developed 12–20 nucleotide, azobenzene-modified “modulators”, in order to arrest the extension of a 34-mer primer by T7 DNA polymerase. The modulators hybridized to the DNA template in the trans form, but UV light-induced trans →
cis photoisomerization sterically disfavored hybridization and thereby allowed primer extension. To increase the photo-modulation efficiency, methods were developed for incorporating multiple azobenzenes into DNA oligonucleotides using a prochiral diol linker. Matsunaga et al. attached multiple azobenzene moieties to a 20-mer DNA oligonucleotide, and monitored the effects of cis–trans isomerization on binding to a complementary RNA strand: $\Delta T_m$ was 4 °C for a single azobenzene, 12 °C for 3 azobenzenes, and 18 °C for 5 azobenzenes. These cumulative effects were relatively small and the overall photomodulation efficiency of RNA degradation with RNase H was low. In order to increase the photo-efficiency, our lab transiently blocked the antisense DNA strand by attaching a complementary sense strand via a single photocleavable linker (PL). Photocleavage was complete within a few minutes using modest UV irradiation, which destabilized the asODN–sODN duplex by 29 °C and led to a 4.4-fold increase in RNase H activity towards a 40-mer RNA substrate.

Other examples of perturbing DNA structure with light include Lewis and Liu’s photoactive DNA hairpins in which the complementary oligos were linked by stilbene, and triple helices formed by an azobenzene-functionalized oligo(thymidine). In recent work by Kröck and Heckel, it was demonstrated that the melting temperature of a DNA duplex could be varied by as much as 21 °C by incorporating three NPE-caged thymidine nucleobases. A single caged thymidine proved capable of stERICALLY blocking transcription by T7 RNA polymerase (Fig. 7). By incorporating one to three guanosines modified with a photolabile 2-(2-nitrophenyl)-propyl (NPP) group, it was also possible to induce G-quadruplex formation with light.

4. In vivo experiments and considerations

A strong case can be made for employing a minimal number of photoactive moieties when developing caged oligonucleotides. Most critically for biological experiments, the irradiation time required to remove $n$ blocking groups that have a quantum uncaging efficiency of 0.5 scales geometrically by $2^n$. Prolonged UV exposures result in greater yields of unintended side products, many of which are radical species that damage DNA and other biomolecules. This likely contributed to the modest results of a recent study in which the hybridization of a 20-mer ODN labeled with an average of 14–16 DMNPE adducts could be photomodulated only between 14% (caged) and 80% (uncaged) of control values. For in vivo studies, low uncaging efficiencies reduce the temporal and spatial resolution of the experiment, due to diffusion of the sample out of the light path. Notably, most of the previously described in vitro studies employed a single photoactive moiety. Biologically active DNA and RNA molecules are often large and photomodulating their activity presents new synthetic and experimental challenges.

Efforts thus far to control gene expression with light in cell and animal studies have relied on “statistical” methods of modifying ODNs, DNA plasmids or mRNA, typically with numerous nitrobenzyl blocking groups. In 1999, Monroe et al. provided the first example of targeting gene expression with light in
a biological system. The phosphate backbone of a GFP plasmid was nondiscriminately labeled with approximately 270 DMNPE caging groups. In HeLa cells that were liposome-transfected with this caged plasmid, GFP was induced in half of the cells, relative to the control caged plasmid, GFP was induced in half of the cells. 

At energy levels above 0.5 J cm\(^{-2}\) light, a significant decrease in levels of GFP expression was observed even in cells containing the wild-type GFP plasmid, which indicated phototoxicity. Control was exerted at the level of transcription, where mRNA production from the caged pGFP template increased 5-fold (3.7% → 19% of control levels) upon photo-activation. Incomplete activation was due presumably to the difficulty of removing most of the DMNPE moieties with non-toxic exposures to UV light.

In 2001, Ando et al. applied similar semi-quantitative photo-uncaging strategies to controlling gene expression in zebrafish embryos, using mRNA and DNA constructs labeled approximately once every 35 bases with a 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) protecting group. A GFP mRNA construct modified with ~30 Bhc groups was tested by in vitro translation: 4% GFP expression was seen with the caged mRNA (indicating very efficient blocking), but only 18% (4.5-fold enhancement) and 23% (~6-fold enhancement) were seen after 10 s and 20 s irradiation with high intensity UV light. Near-native GFP levels were never restored, due to the difficulty of removing the large number of blocking groups with nondamaging UV doses. Similar photomodulation efficiencies were seen within the zebrafish embryo, only in ~50% of the embryos, and required 20 s exposures to UV light. Some variability in embryo microinjections may have contributed to these observations, but it is more likely that mRNA was only partially photo-activated in many embryos, based on the statistical labeling. Indeed, best results were seen when the entire embryo was irradiated, UV light was focused only on large, 100 μm diameter regions. Ando, Okamoto, and associates have worked to refine these techniques and more recently employed caged mRNA to study the role of Lhx2 in zebrafish forebrain growth. Although this method has proven to be somewhat useful, experimental considerations have slowed its widescale adoption by other biological researchers. We anticipate that spatial and temporal resolution will each be improved at least 100-fold when the activity of the DNA and RNA can be modulated using a single photoactive group.

A primary obstacle to the site-specific incorporation of photolabile blocking groups within oligonucleotides has been the incompatibility of these moieties with solid-phase synthesis conditions. In one previous example, the 6-nitroveratraloxycarbonyl (NVOC) group was attached to the N9-position of thymidine and incorporated directly into the oligodeoxyribonucleotide by solid-phase synthesis. Unfortunately, the NVOC group proved to be too stable, requiring irradiation for 5 hours to reveal the antisense oligonucleotide. Methods of making single modifications at the bases, 5' position of the nucleoside sugar backbone, and phosphodiester linkage, proved to be too inefficient or cumbersome for routine applications.

More recently, Heckel et al. have developed a protocol for using “ultramild” phosphoramidites from Glen Research that have protecting groups which can be removed under conditions that are compatible with the nitrophenyl ether cage. Our lab has pursued a different approach that involves functionalizing an amine- or thiol-labeled ODN after solid-phase synthesis. The thiol is most readily placed at the 3' or 5' ends of the oligonucleotide, but an aminothyl-modified base can be placed at any site within the oligonucleotide. These new synthetic methods for site-specific modification provide opportunities for designing caged ODNs that can be activated at near-UV wavelengths for in vivo applications.

5. Future directions

Looking ahead to the future, we consider several areas where advances in the design and application of caged oligonucleotides will yield new biological insights. Based on their ready synthesis and commercial availability, modified DNA and RNA oligos serve as useful scaffolds for proof-of-concept experiments. However, most of these compounds lack nuclease resistance and are not optimized for maximal biological activity with minimal toxicity. We expect that photoactive oligos that incorporate peptide, morpholine, phosphorothioate or other non-natural DNA backbones...
will soon be developed for biological studies. In each case, new synthetic methodologies will be required. In addition, we expect that the field will expand its repertoire of photoactive groups beyond the nitrobenzyl moiety. Other cages provide higher quantum yields at near-UV wavelengths, lower toxicity, and faster rates of photocleavage.36

One rapidly developing area involves the use of caging moieties that can be activated with 2-photon excitation. For biological imaging, there is a useful optical window between 750 and 1000 nm, where there is relatively little absorption from water or endogenous chromophores such as porphyrin-containing hemoglobin. Thus, near-IR light penetrates more deeply into living tissue, which opens a greater range of biomedical applications. In addition, 2-photon excitation provides greater spatial resolution in the z axis, where two IR photons are absorbed nearly simultaneously only in the focal plane.134 In contrast, a focused UV laser generates a cone of light above and below the focal plane, which lowers the axial resolution by expanding the region where photoactivation can occur.

For three-dimensional localized photocleavage, several protecting groups with adequate (~1 Goeppert-Mayer) two-photon cross-sections have been identified, including brominated 7-hydroxycoumarin-4-ylmethyl (Bhc),139,140 nitrobenzofuran (NDBF),141 2-methylthiadenyls,142 NPE-caged coumarin,143 and nitrobenzyl ethers of O-hydroxycinnamates.144 Rapid advances in neurobiology and developmental biology are propelling the next generation of 2-photon imaging and uncaging technologies.145-148 Coupling 2-photon uncaging and imaging will circumvent problems with phototoxicity and depth penetration that are encountered during UV photoactivation.

We expect that cell biology technologies for delivering genetic material will also prove useful for delivering caged oligonucleotides. For example, regionally-directed phototransfection has been demonstrated, whereby genes were delivered to cells using a 405 nm diode laser, as well as a near-infrared, femtosecond pulsed laser.149-151 Regional delivery of caged oligonucleotides, with subsequent UV- or 2-photon activation would provide unprecedented spatiotemporal and quantitative control over gene expression. Delivery of oligonucleotides into mammalian systems remains an ongoing challenge, but new vehicles for presenting oligonucleotides, such as pH-sensitive dendraimers,152 provide opportunities for delivering oligos intravenously and blocking molecular interactions until the desired target is reached. Many experiments will benefit from caged oligos with real-time fluorescent reporters that allow quantification of the in situ concentration of the activated state.99 Finally, laser scanning confocal microscopy can form concentration gradients of photoactivated antisense oligonucleotide that vary throughout a large population of cells. In this way, it will be possible to down-regulate the expression of specific signaling molecules by varying the laser power or dwell time throughout the sample. In conclusion, advances in caged oligonucleotides and photoinitiation methods will soon create new realms for biological exploration.

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