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Caged Substrates Applied to High Content Screening

An Introduction With an Eye to the Future

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Summary

The use of photoremovable protecting groups in biology affords the end user high temporal, spatial, and concentration control of reagents and substrates. High content screening and other large-scale biology applications would benefit greatly from these advantages. Herein, we report progress in this field by highlighting the recent development of controllable siRNA (csiRNA™), which is a dormant siRNA that can be activated using 365 nm light. Two different experimental designs are described to highlight the temporal and concentration variables that can be controlled. First, the RNAi process is activated at two timepoints, 24- and 48-h post-transfection, to demonstrate that the action of csiRNA does not begin until activated. Second, increasing light dosage exposure to cells transfected with csiRNA that controls the concentration of active siRNA molecules. All experiments are conducted in a 96-well format with light delivered through the UCOM™ device.

Key Words: Caged compounds; high-throughput light delivery; light-activated; photoremovable protecting group; siRNA; spatial and concentration control; temporal control.

1. Introduction

Sudden activation of chemical, biological, and physical processes has been a goal in fundamental studies in biology, chemistry, and physics since the initial flash photolysis studies by Nobel Laureate Sir George Porter at the end of World War II *(1)*. A most effective means for rapid activation of such a process has developed through photochemistry by employing photochemical reactions of modified biological substrates or reagents *(2)*, or employing photo activated fluorescent sensors *(3–6)*. These substrates are attached to photoremoveable protecting groups, commonly known as "cages," and are truly effective in altering the chemical and biological activity of the protected substrates. Cages might provide "protection" or inactivation in a number of ways including steric hindrance to the substrate's entrée into tight binding domains or masking the functional group(s) responsible for binding by H-bonding or electrostatic attraction. The location of the attachment of the cage on the substrate, therefore, is of critical importance in that the position of the cage must prevent the normal chemical and biological action of the substrate. Other practical considerations must also be addressed including the photochemical efficiency of the release of the substrate, the available wavelength region for activation of the caging chromophore, and the ease of synthesis or installation of the caged substrate *(7)*.

The advantages of caged substrates are extensive. In addition to control of the temporal release of the substrate through manipulations of the light source, the precise location and the

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

exact quantity of released substrate are experimental parameters that are controlled by the researcher through the choice of the optical light source (from pulsed laser and high-intensity two-photon laser excitation to conventional continuous light sources) as well as the optical and light transmission pathways employed. Furthermore, because light is both a "traceless" and "random access" reagent *(8)*; it leaves no residue that might cause further, deleterious reactions, is indiscriminate in molecular selection; and it provides great versatility in activating caged substrates. The initiation, detection, and detailed study of a great number of biochemical and physiological processes have been successfully pursued *(9,10)*. As an example, caged fluorescent probes have been commercially available for the study of molecular processes for at least two decades *(11)*.

A compilation of caged substrates that illustrate the range and scope of this field would itself be timely but such a list would necessarily be dated. In this growing field, new chromophores and additional substrate candidates for caging are constantly being added. Hence, we choose here to present representative published examples that have stood the test of careful examination and warrant further consideration for the developing field of high content screening (HCS) analysis and imaging. Although many reagents have been "caged" and their release rates, efficiencies, and reaction parameters determined, the examples selected for this chapter (listed in **Table 1**) are restricted to representatives of the range and scope of known biological agonists, antagonists, and inhibitors that have been studied.

There is very limited information on applications of caged compounds in high content and high-throughput screening owing, in large part, to the limits of the instrumentation and techniques necessary for photorelease under the rigorous conditions required for the screening and imaging methodology. The situation is changing, however, as exploratory, innovative applications appear. The most recent applications of caging chemistry include the use of caged fluorophores, which penetrate cell membranes, resulting in high loading of fluorescent precursors within cells for imaging and bioconjugation *(9)*. Other studies, directed toward two-photon decaging, provide more precise spatial control *(10)*. Although these applications are very useful for single cell investigations, they do not address the needs of high-throughput systems, which would greatly benefit from the spatial and temporal control afforded by caged compounds. In addition, caged compounds would open up the possibility of continuous monitoring of responses in cells under repetitious stimulation of a cell after a single transfection step.

To bridge the single molecule caging technology to larger scale biological applications, instrumentation (UCOM, Panomics, Fremont, CA) has recently been developed that delivers precise and uniform lumination over the entire area of a 96-well (or larger) microplate that can release substrates in cells, with a combination of temporal and dose-dependent control and in a format compatible with simultaneous, multiple processes in required in HCS. In the high-throughput mode, the UCOM serves as the essential instrument in development of whole cell assays. One could easily envision an entire series of caged reagents delivered to cells in 96-, 384- or 1536-well microplate format. The temporal and concentration variations are experimentally controlled directly through the UCOM.

It is well understood that HCS systems are designed to yield enormous amounts of information per well, including kinetic measurements of on-/off-response rates, along with selective activation within cellular subdomains. The difficulty for most HCS systems arises from the implementation of substrate application, usually based on pipet introduction of the active reagent or substrate.

The advent of multiple technologies to delivery macromolecules into cellular compartments has been crucial in high content and high-throughput screening and other multiplexed screening systems. Techniques, such as electroporation, are complemented by technologies that involve the covalent attachment of reagents such as TAT *(11)* and antennapedia *(12)* are now commercially available. Complexation reagents such as lipid-based systems (e.g., Lipofecamine and

Table 1
Caged Substrates, Caging Chromophores, and Efficiencies Applied in Biological Studies

Classes of released substrates	Representative examples of substrate	Caging chromophores[a]	Approximately λ_{excit} (nm) ranges[b]	Quantum yields[c] (Φ)	References
Phosphates, nucleotides, and so on	H_3PO_4	pHP	300–340	0.3–0.38	***28***
		DMCM, DMACM	385	0.08	***29***
		BNZ (pH dependent)	300–365	0.01–0.15	***30***
	ATP	pHP	300–340	0.3	***31***
		oNB	300–370	0.19	***32,33***
		oNP	320	0.63	***34***
		DMACM	385	0.07–0.09	***35***
	GTP	pHP	300–330	na	***36***
		oNP	300–350	na	***37***
	Thymidine	oNB, oNP	365	0.2	***38***
		oNBP	350	na	***39***
	cAMP	BNZ	360	0.33	***8,40***
		ACM; MCM	340	0.07	***41***
	NADP	NV	>300	na	***42***
	DNA	oNP	360	na	***43***
	RNA	BHC	350–365	na	***44***
		oNB	308	na	***45***
	siRNA	oNB			***46***
	Phosphopeptides	oNP	300–365	0.26–0.33	***47,48***
C-terminus carboxylic acids	Glu	pHP	>300	0.14	***49***
Amino acids, oligopeptides, and proteins		HCM-carbamate	740 (2 hν)	1 GM	***50***
		MNI	350	0.085	***51***
		N-sub-6-oNP-7-coumaryl-3-carboxyl	300–400 740 (2 hν)	0.33 1 GM	***52***
	GABA	pHP, *m*-substituted pHP analogs	300–390	0.03–0.38	***53***
	Serine	oNP-carbamate	350	0.65	***54,55***
	Aspartate	MNI	334–364	0.09	***56***
		oNB	315	na	***57***
		β-DCNB	308	0.14	***58***
	Alanine				***59***
	NMDA	DNBH	345	na	***60***
	Capsaicin	oNB, NV	300–375	na	***61***
	Ala-Ala	pHP	313	0.26	***62***
	Leu-leu-Me	4-gluco-oNB	375	N/A	***63***
	Acetate	DMBNZ	>300	0.64	***64***
	Bradykinin	pHP	>300	0.22	***38***
	Fluorescein	oNB	350	na	***65***
N-terminus amines, and so on	Phenylephrine	oNB, NV	300–400	0.1–0.4	***66***
Amino acids, oligo-peptides, and proteins	Epinephrine	oNB, NV	300–400	0.1–0.4	***42***
	Isoproterenol	oNB, NV	300–400	0.1–0.4	***42***
	NADP	CNB	>320	0.09–0.19	***67***

(Continued)

Table 1 ***(Continued)***

Classes of released substrates	Representative examples of substrate	Caging chromophores[a]	Approximately λ_{excit} (nm) ranges[b]	Quantum yields[c] (Φ)	References
	Nitrous oxide	5,8-dimethoxy-1-allylnaphthyl, others	350	0.66	***68,69***
Amino acid side chains	C-Kemptide (cysteine)	oNB	300–365	0.62	***70***
	Cysteine, tyrosine	oNB	300–350	na	***71,72***
	Aspartate	DNBH	300–400	0.6	***73***
	Arginine	DMoNB	300–400	0.1–0.4	***74***
	Tamoxifen	NV	365		***75***
Ca^{2+}	EGTA	oNB	347		***11***

[a]Abbreviations for the chromophores are: pHP = *p*-hydroxyphenacyl; oNB = *o*-nitrobenzyl; oNP = *O*-nitrophenethyl; NV = 4,5-dimethoxy-*O*-nitrobenzyl; CNB = α-carboxy-*O*-nitrobenzyl; BNZ = benzoin; DMBNZ = 3′,5′-dimethoxybenzoyl; HCM = 7-hydroxycoumarylmethyl; ACM = 7-acetoxyCM; MCM = 7-methoxyCM; DMACM = 7-dimethylaminoCM; DMCM = 6,7-dimethoxycoumarylmethyl; MNI = 4-methoxy-7-nitroindoline; DNBH = *o,*o′-dinitrobenzhydryl; BHC = 6-bromo-7-hydroxycoumarin-4-ylmethyl.

[b]The wavelength or wavelength range is based on data provided from known UV-vis spectra reported in the references or is estimated based on available data from other sources.

[c]Efficiencies vary with substituents on the chromophore and with changes in the reaction media and conditions. GM = Goppert-Meyer units for two photon (2 hν) excitation.

Oligofecamine) and peptide based systems (e.g., Pep1 ***[13]*** and MPG ***[14]***) are also available to deliver substrates such as oligonucleotides, peptides, and proteins through the cell membrane. Among these, the most effective delivery agents are those that transport the cargo into the cell and avoid endosomal pathways. The Express™ reagent (Panomics) is such a delivery reagent system, which is MPG-based and thus successfully evades endosomal pathways ***(15)***.

Despite the numerous advantages these delivery reagents offer over microinjection or standard pipet techniques, spatial and temporal control of cell activation frequently remains elusive to those implementing in commercially available HCS system. In this aspect, HCS would benefit greatly from a photoactivated caged initiation process.

Batch transfection of caged molecules offers the advantage that equal amounts of silent or inactive antagonists, agonists or substrates can be delivered to all cells and thereby makes the transfection independent of the assay outcome. Thereafter, the uniform illumination to multiple cell arrays with transfected caged reagents under prescribed conditions enables initial null ($t = 0$) measurements followed by precise regulation of the substrate release for HCS. In this way, caged compounds will yield far greater information than simple batch experiments with a group of cells that produce repeatable responses after a period of recovery. This is illustrated with the recent development of controllable siRNA (csiRNA ***[16]***).

Caged siRNA is a timely example because RNAi has quickly become one of the most exciting arenas ***(17)***, owing in large part to its potential in drug discovery and therapeutics ***(18)***. In addition, recent studies have incorporated RNAi into HCS assays ***(19)***. csiRNA is therefore at the forefront of application of caged substrates to large-scale biology.

csiRNA is a caged siRNA that is incapable of catalyzing the normal gene expression knockdown process. The biologically benign caged substrate remains dormant and inactive until absorption of 365 nm light in which siRNA is released. The uncaged siRNA is capable of participating in the normal RNAi process. The following sections will highlight two of the controllable features of caged reagents, temporal, and dosage control, to illustrate the potential use of caged reagents in the high content arena.

1.1. Equipment and Materials

This section lists the materials and equipment needed to conduct gene expression knockdown experiments using csiRNA. Although portions of the experimental design are not described as high throughput, the technology is quite amendable to this technique as well.

2. Materials

1. HEK 293 and HeLa cells (ATCC, Manassas, VA).
2. Growth medium: 10% FBS, DMEM, nonessential amino acids, sodium pyruvate, prepared fresh.
3. PC Phosphoramidite (Glenn Research, Sterling, VA).
4. csiGAPDH™ (Panomics, Fremont, CA), light sensitive, store at –20ºC.
5. 5′-phosphate-GAPDH antisense oligonucleotide (TriLink Biotechnologies, San Diego, CA).
6. GAPDH siRNA negative control (Ambion, Austin, TX).
7. Lipofectamine 2000 (Invitrogen, Carlsbad, CA).
8. Standard annealing solution (Panomics).
9. Clear-bottom, black-wall, 96-well microtiter plates.
10. UCOM Microplate Photoactivator (Panomics).
11. QuantiGene Reagent System (Panomics).
12. QuantiGene Probesets (Panomics).

3. Methods

The application of controllable siRNA (csiRNA) to inhibit gene expression will be described herein under five separate headings:

1. The design and synthesis of csiRNA strands **(Subheading 3.1.)**.
2. The quantum efficiency to establish a working curve for variable gene knockdown and establish the maximum energy required for 100% release of the siRNA's activity **(Subheading 3.2.)**.
3. Delivery of csiRNA into cells cultured in a 96-well format **(Subheading 3.3.)**.

This chapter will describe two different experiments to illustrate two features of csiRNA: temporal control and dosable activation.

1. Light-activation of csiRNA at t = 4 or 24 h post-transfection, followed by gene expression analysis at t = 4, 24, and 48 h **(Subheading 3.4.)**.
2. Increased activation of csiRNA through increasing energy of light, followed by gene expression analysis at t = 24 h post-transfection **(Subheading 3.5.)**.

Finally, we will close with a few concluding remarks **(Subheading 3.6.)**. Throughout the discussion, items that require specific care or particular attention will be described in **Subheading 4.**

3.1. Reagent Preparation

SiRNA oligonucleotides were designed in accordance with guidelines set forth by Tuschl *(20)*. For this discussion, GAPDH was used as the gene of interest. The general structure of siRNA molecules is a double-stranded 21-mer ribooligonucleotide with TT-overhangs on each 3′-terminus. The sense and complementary antisense strand syntheses were carried out using standard phosphoramidite chemistry. The GAPDH negative control siRNAs were obtained from Ambion. The sequence of the GAPDH siRNA sense strand is 5′-caucaucccugccucuacuTT-3′.

The mode of action of siRNA has been well studied *(21)*, and several reports have noted the importance of the phosphorylation of the 5′-antisense strand during gene expression knockdown *(22)*. As such, the 5′-end was targeted for protection with a photoactivatable protecting group (*see* **Note 1**). The photolabile phosphoramidite, [1-*N*-(4,4′-dimethoxytrityl)-5-(6-biotinamidocapro-amidomethyl)-1-(2-nitrophenyl)ethyl]-2-cyanoethyl-(*N*′,*N*′-diisopropyl)-phosphoramidite [obtained from Glenn Research, Sterling, Virginia] was coupled to the 5′ terminus of the antisense strand of a 21-mer siRNA using standard phosphoramidite chemistry during the normal oligonucleotide synthesis. The modified, 21-mer antisense strand was purified using RNase-free

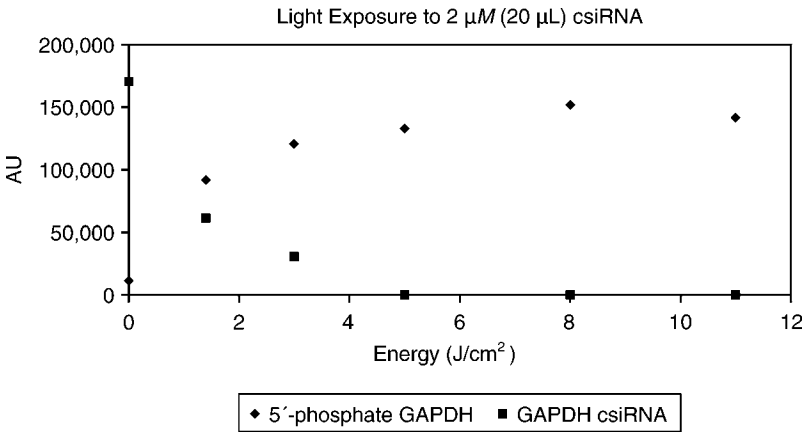

Fig. 1. Light dose–response curve for photorelease of β-actin csiRNA.

HPLC and the purity verified by gel electrophoresis and mass spectrometry (*see* **Note 2**). The sense and antisense strands were annealed:

1. Dissolve the oligonucleotide pellet in standard annealing buffer to a concentration of 300 μ*M*.
2. Confirm the concentration through UV absorption and dilute the sample to 100 μ*M* stock solution (*see* **Note 3**).
3. Combine equal volumes of each oligonucleotide in a 500 μL amber vial.
4. Vortex and centrifuge the sample for several seconds, heat the solution at 85°C for 5 min, and allow the sample to cool to room temperature over 4 h.
5. Vortex and centrifuge the sample for several seconds. The final concentration for the stock solution of annealed csiRNA or siRNA is 50 μ*M*, which is confirmed using UV absorption.
6. Samples might be aliquoted and diluted for working stock solutions.
7. Annealed samples can be stored at –20°C for up to 6 mo and thawed for desired use.

3.2. Analysis

HPLC analysis of the antisense csiRNA was used to establish a light–dosage working curve for csiRNA. Concentration curves of pure starting material (GAPDH csiRNA) and photoproduct (5′-phosphate GAPDH siRNA) were established. Samples of csiRNA were exposed to 365 nm light using the UCOM while monitoring the amount of caged and released csiRNA through HPLC analysis (**Fig. 1**). The energy light flux to uncage 100% of csiRNA at 2 μ*M* was found to be 5 J/cm^2. The initial energy light flux of 1.4 J/cm^2 released approx 26 pmol of csiRNA, which is nine times greater than the amount of csiRNA exposed to cells. For in vivo release of csiRNA cells will be exposed to 1.4 J/cm^2 of 365 nm light **(***see* **Note 5)**.

3.3. Cell Preparation: Transfection of csiRNAs

HEK 293 or HeLa cells were transfected using Lipofecamine 2000 in a 96-well clear-bottom, black-wall microtiter plate in accordance with the csiRNA manual (*see* **Note 4**). Approximately 5000 cells were plated. The final concentration of csiRNA delivered to the cells was 3 n*M*. At $t = 4$ h post-transfection, the complexes were removed and replaced with 120 μL of fresh complete growth medium (*see* **Note 6**).

3.4. Preliminary Studies: GAPDH Expression Knockdown Through csiRNA Activation at Different Time-Points

A key advantage of caged materials is the ability to keep the substrates silent until it is required, experimentally, to activate the substrate. Here, this feature is demonstrated with

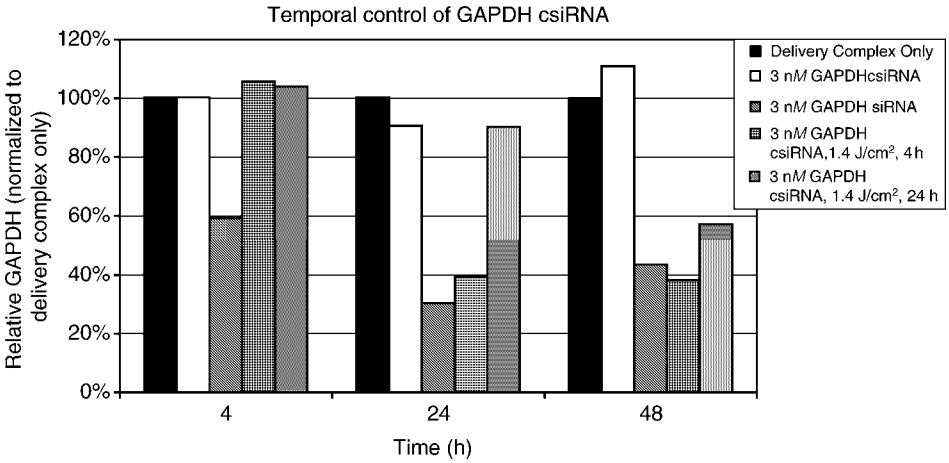

Fig. 2. GAPDH expression knockdown at various time-points. GAPDH csiRNA was transfected into HeLa cells and exposed to 365 nm light at $t = 4$ or 24 h posttransfection. Control conditions include cells exposed to transfection reagent only, GAPDH csiRNA without light activation, and GAPDH siRNA. Expression levels were measured at $t = 4$, 24, and 48 h for all conditions. Control experiments (delivery complex only and csiRNA without 365 nm light exposure) shows GAPDH expression continues unimpeded. GAPDH expression levels are knocked down for GAPDH siRNA and for csiRNA *only* after exposure to 365 nm light.

csiRNA by transfecting cells as described in **Subheading 3.3.** and incubating cells for 4, 24, or 48 h posttransfection. Cells were exposed to 365 nm light using UCOM, according to the UCOM user manual, at $t = 4$ or 24 h time-points. GAPDH expression levels were analyzed using Quantigene, according to the user manual (**Fig. 2**).

Cells that were not transfected with GAPDH siRNA or csiRNA maintained their normal GAPDH expression levels. Cells that were transfected with csiRNA maintained their normal GAPDH expression levels until csiRNA was activated with the UCOM. The most important advantage is allowing substrates to remain dormant in cells until the desired time to activate them, illustrated with cells irradiated at 24 h. Prior to irradiation, GAPDH expression levels were normal. However, at $t = 24$ h, cells were exposed to 365 nm light and GAPDH expression was knocked down ($t = 48$ h) to less than 60% below normal GAPDH levels.

3.5. Light Dosable Photo-Activation of csiRNA

The importance of controlling dose release in biological studies cannot be overstated (*see* **Note 6**). Kinetic studies, as well as phenotypic assays, are greatly enhanced when modulators introduced to cells can be activated with a high degree of accuracy and precision. For most phenotypic assays, especially, it is highly desirable to accurately titrate the amount of material required to elicit a phenotypic response. In this context, we demonstrate the activity of csiRNA can be tuned by controlling the energy exposed to transfected cells. By exposing the cells to increasing light energy, an increase in siRNA activity is achieved. The beauty of this system (as with all caged systems) is that a known number of photons (i.e., energy) will trigger a known quantity of siRNA precisely because there is a single caging group positioned at the 5′-end per siRNA molecule.

Cells were prepared as previously described in **Subheading 3.3.**

1. The cells were exposed to 0.0–1.4 J/cm^2 of 365 nm light using a UCOM Microplate Photo-Activator (Panomics) according to the UCOM manual.
2. The cells were incubated at 37ºC for $t = 24$ h post-transfection, and lysed using QuantiGene lysis buffer according to the QuantiGene user manual. Replicates of three wells were run for all conditions tested. Gene expression levels were measured using QuantiGene according to the user manual.

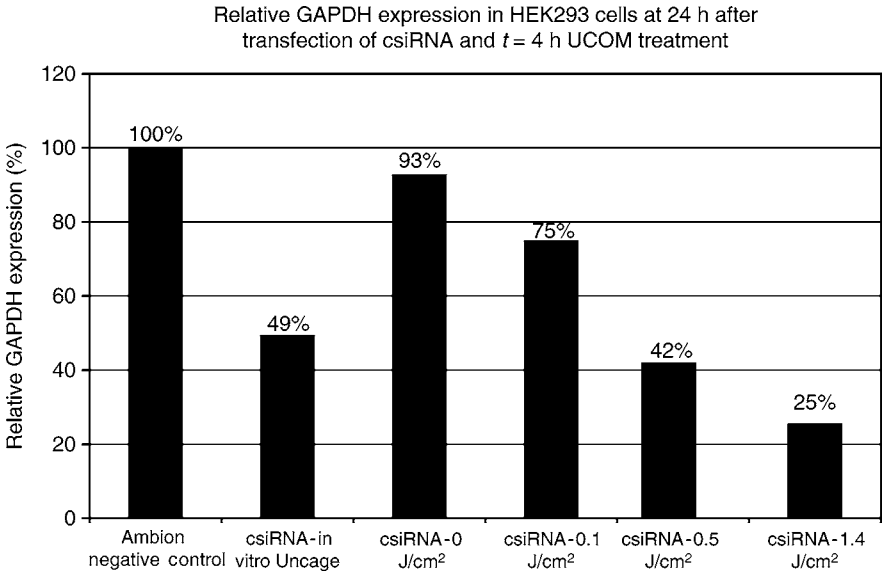

Fig. 3. In vivo light–dosage exposure to HEK 293 cells transfected with GAPDH csiRNA, negative control siRNA at $t = 4$ h post-transfection. GAPDH csiRNA that was previously exposed to 1.4 J/cm^2 365 nm light was also transfected into HEK 293 cells as a positive control. Cells were incubated at 37°C for $t = 24$ h and the GAPDH expression levels were measured.

As **Fig. 3** illustrates, increasing light dosage results in more csiRNA uncaged to release active siRNA effectively reducing residual mRNA levels through the normal RNAi pathway *(23–27)*. The activity of caged reagents is not simply "on" or "off." By exposing the appropriate energy dosage on the UCOM it is possible to tune in the amount of active reagent available in cells.

3.6. Concluding Remarks

There are an infinite number of applications for including cell survival, cell cycle regulation and cell development. Caging technology offers experimentalists a wide array of control in temporal, spatial, and concentration parameters. And with the advent of tools designed to bring light control to multiplexed assay systems, caged compounds may now be implemented in high content and high-throughput screens. We are, in fact, witness to several technologies that have been available for quite some time, be integrated in complementing fashion. These integrated technologies will surely help to better understand cellular pathways, off-target and downstream effects, and substrate effects on these pathways.

4. Notes

1. Attachment of photolabile groups to RNA and DNA using postsynthetic methods has been reported in literature; however this method does not take siRNA active sites into account. The design described here requires only a single caging group per siRNA molecule to take full advantage of the caging agent (*vide supra*). The postulated methodic placement of the caging group on siRNA molecules is limited to the following locations: 5′-, 3′-, or 2′-hydroxy groups; on the phosphate backbone; or on an individual nucleotide base. It was hypothesized that the 5′-hydroxy group was the most accessible synthetically and would cause the greatest disruption to the RNAi process. To test this, a GAPDH siRNA was synthesized with derivatives that permanently modified 5′-terminus. 5′-*O*-methyl siRNA analogs were shown to have zero activity compared to normal siRNA analogs. In addition, an *O*-alkyl phosphate modified siRNA (5′-C6-amine-GAPDH) also failed to catalyze gene expression knockdown for GAPDH. These experiments indicated a caging group on the 5′-phosphate would also block siRNA action. There are reports of other photoactivatable siRNA systems, which do not cage the 5′-end

Table 2
Purity of Antisense Csirna™ Compared to Background Gene Expression Knockdown: The Lower the Purity of the Antisense Strand, the Higher the siRNA Activity

Lot no.	Purity of antisense strand (%)	GAPDH expression level (normalized to cyclophilin expression levels [%])
1A	99.4	91
2B	97.1	80
3C	96.9	73
4D	96.3	70
5E	93.8	74
6F	78	44

exclusively *(28)*. These systems are not as potent for a number of reasons. Either there are more caging groups per siRNA molecule leading to decreased sensitivity to light, and hence less dosable, or siRNA is caged at random locations. The end result is a system that is not completely silent and might posses some or all normal activity. In addition, to remove all of the caging groups requires substantially more light energy, which can lead to cell death *(75)*.

2. Highly purified csiRNA antisense strands are extremely important for successful controllable knockdown experiments. We investigated the correlation between the purity of csiRNA and the activity of csiRNA in cells, measured by HPLC chromatograms of the antisense strand. Six different lots of various purities were transfected into HeLa cells according to the csiRNA manual and incubated for 24 h at 37°C. The cells were not exposed to 365 nm light in order to keep the csiRNA caged and unreactive. The cells were lysed and GAPDH mRNA expression levels were measured using Quantigene detection system. Any drop in GAPDH expression levels prior to light activation is viewed as a less efficient csiRNA. According to **Table 2**, there is a drop in caging efficiency below 97% purity, and an even more dramatic drop below 94% purity. This is most likely because of (n–1) residues that make up the majority of impurities from oligonucleotide syntheses. For csiRNA (n–1) residues are fully active, complete siRNA molecules.
3. Spectroscopic determination of concentration was carried out for two purposes. First, a 1:1 ratio of sense to antisense should be used to achieve the highest activity. Second, an accurate measure of csiRNA concentration is needed to yield optimal delivery to cells. This will result in the highest potential knockdown activity with the lowest background.
4. It is essential to use clear-bottom microtiter plates, as the UCOM Microplate Photoactivator delivers light from the bottom. The UCOM has been tested to be compatible with the following microtiter plates:
   a. Corning, Costar® (cat. no. 3904).
   b. BD, Falcon (cat. no. 353948).
   c. Greiner (cat. no. 655090).
   d. Nunc, Nalgene (cat. no. 237105).
5. Cytotoxicity experiments for UCOM 365 nm light exposure on HeLa cells showed ED50 values of 21 J/cm^2. It is vital to maintain energy doses lower than the ED50 level. Detrimental effects to cells, including cell death, are evident above the ED50 level. Cells show a very good tolerance to 365 nm light at energy levels below the ED50 value.
6. It is vital that the media be replaced following the transfection protocol. Although transfection methods might be highly efficient, it is impossible to have delivered all csiRNA into the cells. As light is completely unselective toward csiRNA inside or outside of cells, it is necessary to remove the undelivered extra cellular caged reagents.

Acknowledgments

We would like to thank Frank Witney, Gary K. McMaster, and Quan Nguyen for helpful advice and discussions. Support from the National Institutes of Health (Grant no. GM72910 [RSG]) is gratefully acknowledged.

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