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Published on Web 12/16/2010

Patterning of Gene Expression Using New Photolabile Groups Applied to Light Activated RNAi

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Abstract: The spacing, timing, and amount of gene expression are crucial for a range of biological processes, including development. For this reason, there have been many attempts to bring gene expression under the control of light. We have previously shown that RNA interference (RNAi) can be controlled with light through the use of siRNA and dsRNA that have their terminal phosphates modified with the dimethoxy nitro phenyl ethyl (DMNPE) group. Upon irradiation, these groups photolyze and release native RNA. The main problem with light activated RNA interference (LARI) to date is that the groups used only partially block RNA interference prior to irradiation, thus limiting the utility of the approach. Here, we describe a new photocleavable group, cyclo-dodecyl DMNPE (CD-DMNPE), designed to completely block the interaction of duplexes with the cellular machinery responsible for RNA interference prior to irradiation. This allowed us to switch from normal to a near complete reduction in gene expression using light, and to construct well-defined patterns of gene expression in cell monolayers. Because this approach is built on the RNA interference pathway, it benefits from the ability to quickly identify duplexes that are effective at low or subnanomolar concentrations. In addition, it allows for the targeting of endogenous genes without additional genetic manipulation. Finally, because of the regiospecificity of CD-DMNPE, it allows a standard duplex to be quickly modified in a single step. The combination of its efficacy and ease of application will allow for the facile control of the spacing, timing, and degree of gene expression in a range of biological systems.

Introduction

We and others are pursuing the control of gene expression using light, as this will allow for the spacing, timing, and degree of gene expression to be manipulated.[1−9] These features are key components of many biological phenomena; therefore, such a tool would have applicability to the analysis of diverse processes such as development and neuroplasticity. RNA interference (RNAi) is particularly appealing as a basis for light controlled gene expression for multiple reasons: (1) It is relatively simple to identify effective siRNA and dsRNA sequences to target any gene. (2) No additional genetic manipulation of target organisms/tissues are necessary. (3) Small amounts of material are required (pM in the case of dsRNA).

For this reason, our lab has pioneered light activated RNA interference (LARI).[7,10,11] Subsequently, others have also described related attempts to control RNAi with light.[4,6] We have shown that the classically used photolabile reagent, diazo-DMNPE,[12] can modify siRNA and results in a partial variation of RNAi with light.[7] Specifically, RNA interference is only partially blocked prior to irradiation. In addition, specific 5′ antisense phosphate modifications by a range of chemically similar reagents suffer from this same incomplete block of RNAi.[11] We have also shown that diazo-DMNPE has a remarkable regiospecificity for terminal phosphates, and that we can use this to direct the incorporation of four DMNPE groups per duplex, by incorporating four terminal phosphates in a duplex. Using this knowledge, we previously improved the block of RNA interference prior to irradiation, using 27-mer blunt ended dsRNA precursors of siRNA which had four terminal phosphates incorporated.[10] However, although improved, this system also suffers from incomplete block of RNAi prior to irradiation (e.g., ∼35% knockdown even in absence of light). This incomplete block severely limits the utility of our

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10.1021/ja107226e

approach, and so we have sought to address this through the synthesis and incorporation of new photolabile groups into dsRNA. The guiding hypothesis is that these groups, prior to irradiation, block interaction with Dicer, and hence prevent RNAi until irradiation removes them. The incomplete block of RNAi prior to irradiation can be due to multiple factors: (1) the groups, while lowering the affinity of Dicer for the modified RNA, do not completely abrogate binding, or (2) the groups can be cleaved by nucleases that remove terminal nucleotides from the modified RNA.

For this work, we designed new photolabile groups that had the potential to have a greater ability to block interactions with Dicer and/or nucleases. This was achieved by incorporating a carboxyl synthetic handle into DMNPE that allowed a range of bulky amines to be incorporated. These bulkier photolabile groups then have the potential to have greater steric clash with Dicer and/or nucleases. We demonstrate that one of these groups, cyclo-dodecyl DMNPE or CD-DMNPE, results in a modified dsRNA that allows gene expression to be switched from full expression to native-like knockdown using light. Using this modified RNA, we were able to construct distinct patterns of gene expression in monolayers, including gradients.

Experimental Section

***tert*-Butyl 2-(4-acetyl-2-methoxyphenoxy)acetate (Compound 1).** Compound **1** was synthesized as described in the literature.[13]

2-(4-Acetyl-2-methoxy-5-nitrophenoxy)acetic Acid (Compound 2). Compound **2** was synthesized as described in the literature.[13]

2-(4-Acetyl-2-methoxy-5-nitrophenoxy)-*N*-cyclododecylacetamide (Compound 3). Compound **2** (50.0 mg, 186 μmol), cyclododecylamine (68.1 mg, 372 μmol), and 1-hydroxybenzotrizole hydrate (56.9 mg, 372 μmol) were dissolved in 900 μL of dimethylformamide (DMF). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (59.6 mg, 311 μmol) was added to the resulting solution and allowed to shake for 15 h. The product was purified by partitioning the reaction mixture between ethyl acetate (10 mL) and saturated sodium chloride (10 mL). The ethyl acetate layer was washed twice with saturated sodium chloride solution and the combined aqueous layers were washed once with ethyl acetate. The combined organic layers were washed with saturated sodium bicarbonate solution, 1 N HCl, and again with saturated sodium bicarbonate solution. The organic layer was then dried with magnesium sulfate and evaporated to give an off-white solid. Yield 63.3 mg (78.4%), TLC (EtOAc/MeOH, 75:25 v/v): $R_f = 0.60$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.95 ppm (d, $J = 8.4$ Hz, 1H), 7.54 (s, 1H), 7.26 (s, 1H), 4.67 (s, 2H), 3.95 (s, 3H), 3.88−3.95 (m, 1H), 2.52 (s, 3H), 1.50−1.64 (m, 4H), 1.15−1.41 (m, 18H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 199.3, 165.8, 153.5, 147.8, 137.6, 132.1, 109.9, 108.7, 67.7, 56.6, 44.1, 30.0, 29.8, 23.3, 23.1, 22.8, 21.3; UV/vis (DMSO): λ_{max} (ε_λ): 262 nm (8037 M^{-1} cm^{-1}), 343 nm (4500 M^{-1} cm^{-1}); MS (*m*/*z*): $[M]^+$ calcd for $C_{23}H_{34}N_2O_6$, 435.2; found, 435.5; reversed phase HPLC−MS (flow rate 0.4 mL/min, runtime 30 min, injection volume 25 μL) solvent A (0.1% formic acid in H_2O), solvent B (0.1% formic acid in acetonitrile (ACN)), gradient 50% B to 100% B over 10 min, isocratic 100% B for 17 min, 100% B to 0% B over 3 min, C_8 Hypersil column (5 μm, 100 × 4.6 mm, Varian): retention time (min) 12.37; ESI−MS (*m*/*z*): $[M]^+$ calcd for $C_{23}H_{34}N_2O_6$, 435.2; found, 435.2.

(*E*/*Z*)-*N*-Cyclododecyl-2-(4-(1-hydrazonoethyl)-2-methoxy-5-nitrophenoxy)acetamide (Compound 4). Compound **3** (32 mg, 73.6 μmol) was dissolved in 2 mL of a 1:1 mixture of acetonitrile and 95% ethanol. Hydrazine monohydrate (71.2 μL, 1.47 mmol) and glacial acetic acid (6.6 μL, 110.4 μmol) were added to the solution and the mixture was refluxed for 4 h at 90 °C. The resulting yellow oily solution was evaporated to dryness and partitioned between chloroform and water (2 mL each). The organic layer was washed twice with water and the combined aqueous layers were washed once with chloroform. The combined chloroform layers were evaporated to dryness yielding a yellow solid. The resulting solid was dissolved in dichloromethane (DCM; 2 mL) and then purified using silica gel flash column chromatography with 5% methanol in DCM. Fractions were collected, dried, and analyzed by LC−MS and appropriate fractions were combined and used for the next reaction. The crude contained a mixture of 2 isomers, E and Z. Yield 32.4 mg (98.2% yield). TLC (EtOAc/MeOH, 75:25 v/v): $R_f = 0.64$; ^{1}H NMR (400 MHz, DMSO-d_6, E or Z isomers, ratio of isomer 1/isomer 2 = 1:3): isomer 1 [δ 7.93 (d, $J = 8.8$ Hz, 1H), 7.66 (s, 1H), 6.89 (s, 1H), 5.43 (s, 2H), 4.63 (s, 2H), 3.92 (m, 4H), 2.06 (s, 3H), 1.46−1.58 (m, 4H), 1.15−1.46 (m, 16H), 0.91−0.96 (m, 2H)]; isomer 2 [7.88 (d, $J = 8.8$ Hz, 1H), 7.39 (s, 1H), 6.99 (s, 1H), 6.41 (s, 2H), 4.59 (s, 2H), 3.90 (m, 4H), 2.55 (s, 3H), 1.46−1.58 (m, 4H), 1.15−1.46 (m, 16H), 0.91−0.96 (m, 2H)]; ^{13}C NMR (100 MHz, DMSO-d_6): only isomer 2 peaks visible δ 197.5, 166.1, 151.5, 145.3, 127.1, 115.4, 111.0, 99.2, 66.9, 43.8, 29.8, 27.6, 23.4, 23.2, 22.7, 22.4, 21.5; UV/vis (DMSO): λ_{max} (ε_λ): 263 nm (7323 M^{-1} cm^{-1}), 346 nm (4470 M^{-1} cm^{-1}); reversed phase HPLC−MS (exact conditions as used for compound **3**): retention time (min) 13.32, 13.90; ESI−MS (*m*/*z*): $[M]^+$ calcd for $C_{23}H_{36}N_4O_5$, 449.3; found, 449.3.

***N*-Cyclododecyl-2-(4-(1-diazoethyl)-2-methoxy-5-nitrophenoxy)-acetamide (Compound 5).** Compound **4** (10 mg, 22.3 μmol) was dissolved in 500 μL of dry dimethyl sulfoxide and protected from light. Manganese(IV) oxide (10 mg, 115 μmol) was added to 75 μL of this solution and shaken gently for 45 min, while protected from light. The characteristically red-orange mixture was centrifuged using a microcentrifuge and the supernatant was filtered through Celite 545 supported by glass wool in a glass pipet. This pad was washed with 58.8 μL of dimethyl sulfoxide to give a final diazo concentration of 25 mM. Compound **5** was freshly prepared each time to cage dsRNA. As per the use of diazo-DMNPE, compound **5** was not isolated or further characterized, beyond its UV−visible spectrum. The UV−vis analysis showed peaks at 280, 346, and 450 nm.

Caging of dsRNA. Caging of dsRNA with diazo-DMNPE was performed following our previously described methods.[7] Caging with compound **5** (diazo-CD-DMNPE) was done similarly with the following modifications: To 250 μL of 50 μM dsRNA in tris-acetate-EDTA buffer, freshly prepared compound **5** in DMSO (50 μL, 1.25 μmol) was added and gently shaken for 12 h, protected from light. The addition of freshly prepared compound **5** in DMSO (50 μL, 1.25 μmol) was repeated every 12 h for four times. Finally, a double amount of compound **5** in DMSO (100 μL, 2.50 μmol) was added and allowed to react for 24 h, protected from light.

Purification of Caged dsRNA. As previously described.[10]

Mass Spectrometry of Caged dsRNA. As previously described.[10]

Cell Culture and Irradiation. As previously described,[10] with the following modifications: HeLa cells were plated in a 96 well format, at 70% confluency, and allowed to culture for 18−20 h. Cells were then co-transfected with pEGFP-C1 (0.099 μg/well), pDsRed2-N1 (0.132 μg/well) plasmids, uncaged or caged dsRNA (0.20 pmol, 1.56 nM for 1× concentration and 0.98 pmol, 7.80 nM for 5× concentration), and lipofectamine (1.13 μL/well) in Opti-MEM (120 μL/well) for 6 h. Cells were washed with Opti-MEM (150 μL) and irradiated for 10 min in Opti-MEM (100 μL) using a Blak-Ray lamp (Model XX-15 L, 30 W) placing the lamp 10 cm above the 96-well plate. One half of the plate-lid was protected from light using aluminum foil and the other half was exposed to UV light filtered through a WG-320 long pass filter (Edmund Optics). Cells were then cultured in antibiotic free Dulbecco's Modified Eagle Medium (DMEM; 200 μL) containing fetal bovine serum (FBS; 10%) for 42 h and washed with phosphate buffered

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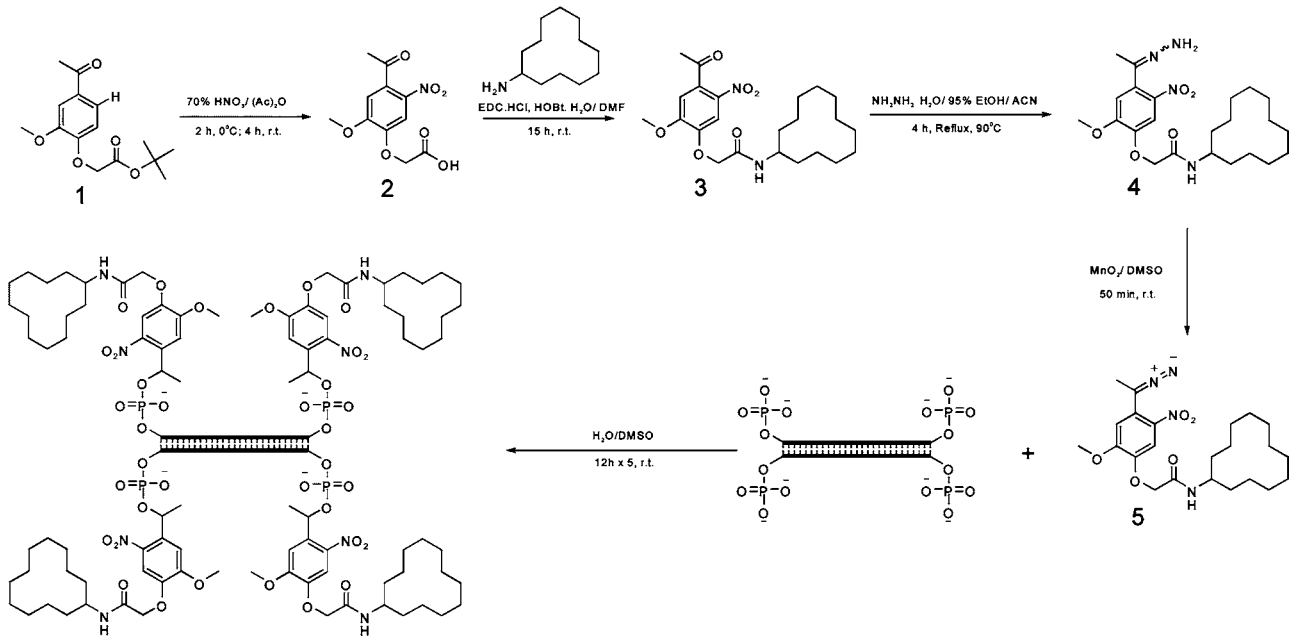
Scheme 1. Synthesis of dsRNA Modified with Four CD-DMNPE Groups



saline (PBS; 200 μL). After adding fresh PBS (100 μL), the fluorescence intensity of both GFP and RFP were determined using a fluorescent microplate detector (Beckman Coulter). Each point was corrected for mock (cells treated with lipofectamine in Opti-MEM) and then each GFP/RFP ratio was normalized to the GFP/RFP ratio of the control (plasmids and lipofectamine in Opti-MEM, unirradiated).

Half-Well Patterning Experiment. A mask was printed in a 96-well format with a line on the diameter of each well using a monochrome laser printer. A flat-black painted brass metallic strip was then aligned with its edge on the diameter to cover one-half of each well in the column. This metallic mask was aligned and glued to the bottom of a 96-well plate with the nonmetallic side in contact with the plate. Using this plate, cell culture and irradiation was performed as above except that the mode of irradiation was switched from above the plate to below. Lamp, time of irradiation, and distance of the lamp from the cells, as well as all cell culture conditions were identical to those used for the whole well experiments.

Gradient Patterning Experiment and Analysis. A mask with a linear gradient in each well of a 96-well plate was printed on a Highland-903 transparency using a color laser printer (Canon). The gradient was printed within a rectangular area of the size of field of view of the microscope in order to visualize the complete gradient in a single view. The mask was aligned and glued to the bottom of a 96-well plate with the nonprinted side in contact with the plate. Using this designed plate, cell culture was performed in an identical manner as the half-well experiment with an exception of the source of irradiation. In this experiment, the 96-well plate was irradiated from the bottom for 10 min in the tissue culture hood using the point source, described in the text. Following microscopy, image analysis was performed using Adobe Photoshop by calculating the mean intensity (A) of equally divided sections (n) and by calculating the background intensity (B). For each section, the mean intensity was corrected for its background intensity ($A_n - B$) and then each section was normalized to the corrected intensity of the first section ($A_1 - B$), that is, $(A_n - B)/(A_1 - B)$. This was performed for both GFP and RFP to produce values described by $[\{(A_n - B)/(A_1 - B)\}_{GFP}]/[\{(A_n - B)/(\mathbf{A}_1 - B)\}_{RFP}]$. These were then plotted as a function of distance in the image.

Letter Patterning Experiment. A mask in a 96-well format with letters in each well was printed on a Highland-903 transparency using monochrome LaserJet printer (HP). To avoid any leakage of light through the black printed mask at the microscopic level, Magic Dark Solution (Advance Chemical Engineering, Inc.) was applied to the transparency following the manufacturer's instructions. Each mask was separately aligned and glued to the bottom of a 96-well plate as given above. Using this plate, cell culture and irradiation were performed in the same manner as the gradient patterning experiment.

Microscopy. Microscopy was performed using a Nikon Eclipse TE-300 inverted epi-fluorescence microscope. All images were taken using the 2× objective lens. GFP and RFP filter cubes were used for GFP and RFP signals, respectively. For fluorescence images, 50 and 15 s of exposure time were used for GFP and RFP, respectively. The monochrome images generated using CCD were pseudocolored to the appropriate color.

Cell Viability Assay. To see if there was any cytotoxicity due to irradiation, cell viability was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). The standard cell culture conditions were followed as described above including plating, transfection, and irradiation. At 42 h post-irradiation, cell viability was measured by following the manufacturer's protocol. Each absorbance value was normalized to cells treated with lipofectamine and unirradiated.

Results

CD-DMNPE was synthesized starting from the nitro-keto-acid originally described by Holmes.[13] This was condensed with cyclododecyl amine, converted to the hydrazone using hydrazine, and then oxidized to the diazo compound using MnO_2 (synthetic details may be found in the Experimental Section and Supporting Information). We have previously shown that terminal phosphates of nucleic acids have much greater reactivity toward diazo-DMNPE than do internal phosphates, so we can regularly incorporate exactly four groups per duplex. This diazo form of CD-DMNPE was then reacted with a 27-mer blunt end GFP targeting dsRNA containing four terminal phosphates (Scheme 1). A gel of the modified and HPLC purified RNA as well as its ESI−MS are shown in Figure 1. We observe two groups of CD-DMNPE incorporated in each strand, consistent with our understanding of the regiospecificity of diazo-DMNPE. (Characterization of the scrambled GFP targeting modified duplex used can be found in Supporting Information.)

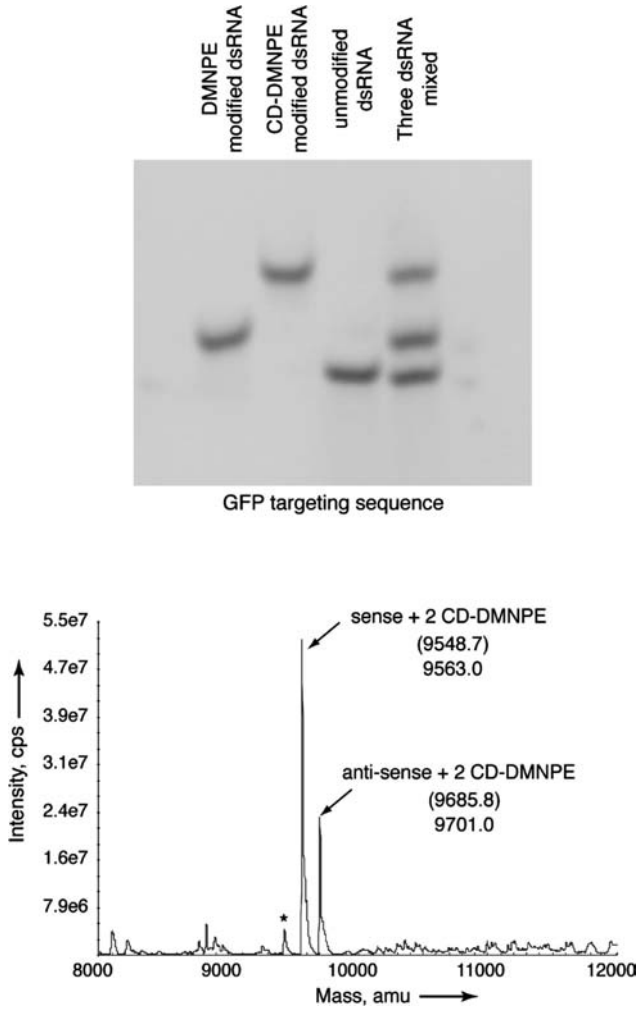


Figure 1. (Top) Nondenaturing PAGE gel of purified, CD-DMNPE modified dsRNA, DMNPE modified dsRNA and unmodified dsRNA. The fourth lane is these three samples, co-loaded. (Bottom) Deconvoluted ESI−MS of tetra-CD-DMNPE modified dsRNA. Each modified strand is visible, and the expected (parenthesis) and actual masses indicated. The asterisk (*) indicates a depurinated specie.

We tested the efficacy of this modified RNA at controlling RNA interference with light using our high throughput gene expression assay.[7] HeLa cells were transfected with GFP and RFP expressing plasmids, as well as dsRNA at 1.56 nM. This dsRNA was CD-DMNPE modified, blunt end 27-mer that targets GFP.[14] Control duplexes also tested were (1) CD-DMNPE modified, scrambled 27-mer; (2) unmodified 27-mer targeting GFP; (3) unmodified, scrambled 27-mer; and (4) plasmids alone, with no dsRNA. In addition, to compare the efficacy of the new groups to our previously used DMNPE group, we simultaneously analyzed the DMNPE modified, blunt end 27-mer that targets GFP. After 6 h of transfection, cells were washed, fresh media was applied, and the cells were either irradiated (using a 320 nm long pass filtered Black-ray bulb for 10 min) or not. They were then allowed to culture for 42 h. GFP and RFP signals were then read using a fluorescent plate reader, and the ratio of GFP to RFP (corrected for cell autofluorescence, typically a small number) was normalized to that ratio in the control GFP and RFP plasmids-only cells. We

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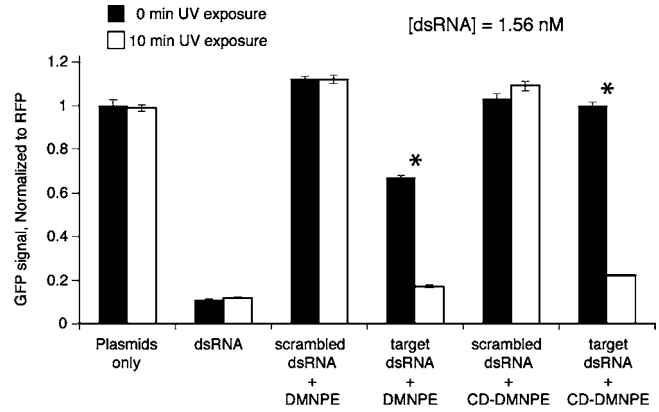

Figure 2. Light activated RNA interference comparing CD-DMNPE with DMNPE modified dsRNA at 1.56 nM. All samples were transfected with GFP and RFP expressing plasmids. "Target dsRNA" refers to a GFP targeting dsRNA, whereas "Scrambled dsRNA" is this sequence randomized. "+ DMNPE" or "+ CD-DMNPE" refers to these duplexes tetra-modified with the indicated photolabile group. All results are the average of 5 different determinations. Asterisks indicate conditions in which light initiated a significant change in expression ($p < 0.01$).

confirmed that there is no phototoxicity induced by our irradiation conditions. Cell viability, as determined by the MTT assay, was identical when cells were irradiated or unirradiated (see Supporting Information).

The results are shown in Figure 2. Unmodified dsRNA that targets GFP strongly knocks down GFP expression, independent of light. Unmodified scrambled dsRNA maintains expression similar to plasmids alone, as does the CD-DMNPE modified scrambled sequence. CD-DMNPE modified GFP targeting dsRNA, however, gives a strong, light dependent triggering of gene expression reduction, that is significantly improved relative to the DMNPE modified dsRNA (a change of expression with light of 1.0 to 0.22 versus 0.67 to 0.17). We observed that the post-irradiation knockdown of our CD-DMNPE sample was close to but not quite at the native level of knockdown for unmodified dsRNA (0.22 versus 0.12). To increase this level of knockdown, we surmised that increasing the concentration of modified dsRNA might be effective, as the bulkier group might maintain complete block, but allow a higher level of deprotected dsRNA to be released upon irradiation. To test this, we performed the identical experiment at a concentration of 7.8 nM RNA. The results are shown in Figure 3.

We see that the performance of the DMNPE modified RNA has diminished, with preirradiation GFP levels being more substantially knocked down. The range for the CD-DMNPE modified sample however has improved. The range is now 0.97 to 0.11 (pre- to post-irradiation). This now approximates an almost ideal light switch for gene expression. The data in this experiment are highly reproducible. We repeated these experiments using a dsRNA that targets RFP expression, comparing unprotected, DMPNE protected, and CD-DMNPE protected dsRNA. As with GFP, the CD-DMNPE protected dsRNA improved the window of expression varied with light relative to DMNPE-protected dsRNA (see Supporting Information).

With this method in place, we then examined the applicability of it to making patterns of gene expression in cell monolayers. The ability to do this has the potential to make significant contributions to the study of development, where developmental outcome for a specific cell is closely linked to the spacing, timing, and amount of gene expression. We used the same GFP targeting and scrambled dsRNAs used in the studies described

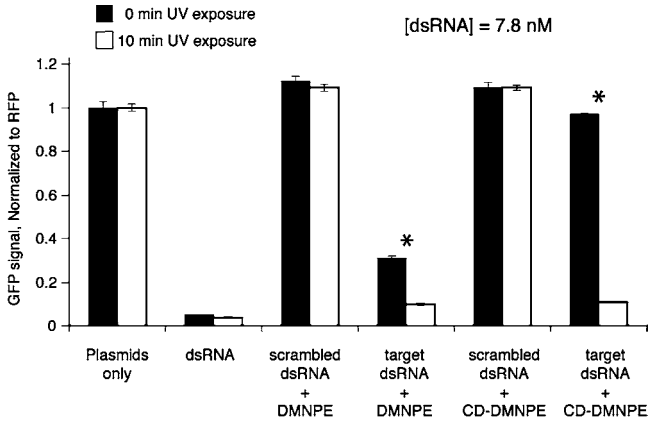

Figure 3. Light activated RNA interference comparing CD-DMNPE with DMNPE modified dsRNA at 7.8 nM. All samples were transfected with GFP and RFP expressing plasmids. "Target dsRNA" refers to a GFP targeting dsRNA, whereas "Scrambled dsRNA" is this sequence randomized. "+ DMNPE" or "+ CD-DMNPE" refers to these duplexes tetra-modified with the indicated photolabile group. All results are the average of 5 different determinations. Asterisks indicate conditions in which light initiated a significant change in expression ($p < 0.01$).

above. Each well was masked with a piece of 400 μm thick brass that was painted black to limit additional reflections of light. This was positioned on the base of the 96 well culture plate so that half of each examined well was masked from direct light exposure. Because this light source was not collimated, nor a point source, we rely on the closeness of the mask to the cells to create a sharp block of light. Even with this, we observe some light leakage at the edge of the mask.

As before, cells were transfected with GFP and RFP plasmids as well as experimental or test dsRNA duplexes. The following were the tested species: (1) plasmids only (no RNA), (2) GFP targeting dsRNA, (3) scrambled dsRNA, (4) CD-DMNPE protected, scrambled dsRNA, and (5) CD-DMNPE protected, GFP targeting dsRNA. After 6 h of transfection, the media was replaced and cells were irradiated from below using the same lamp used previously. Cells were allowed to culture for 42 more hours. At this point, the mask was removed and the patterns of GFP and RFP expression were examined using epifluorescent microscopy and GFP or RFP filter cubes. All images were acquired using the identical exposure for each fluorescent channel. Pseudocoloring of the black and white images to the appropriate color was done, consistent with the fluorescent channel used. Aside from this, no other processing was done.

The resultant patterns are shown in Figure 4. For the plasmids only wells, the GFP and RFP signals are uniform and strong. The GFP targeting dsRNA uniformly reduces GFP but not RFP expression. The scrambled dsRNA and the CD-DMNPE caged, scrambled dsRNA have no effect on either GFP or RFP. These four controls indicate that irradiation alone is not affecting gene expression and that the release of the CD-DMNPE group is not somehow differentially affecting gene expression. The CD-DMPNE caged, GFP targeting dsRNA however produces a strong pattern of expression, with GFP signal strongly reduced where light exposure took place, and full GFP expression observed in the adjacent, masked area. The untargeted RFP signal in this same area shows uniform expression with no such pattern.

The results in Figure 4 are representative of the pattern obtained for CD-DMNPE modified GFP targeting dsRNA. Two other replicates of this from the same experiment are shown in Supporting Information, and show the identical pattern. We have

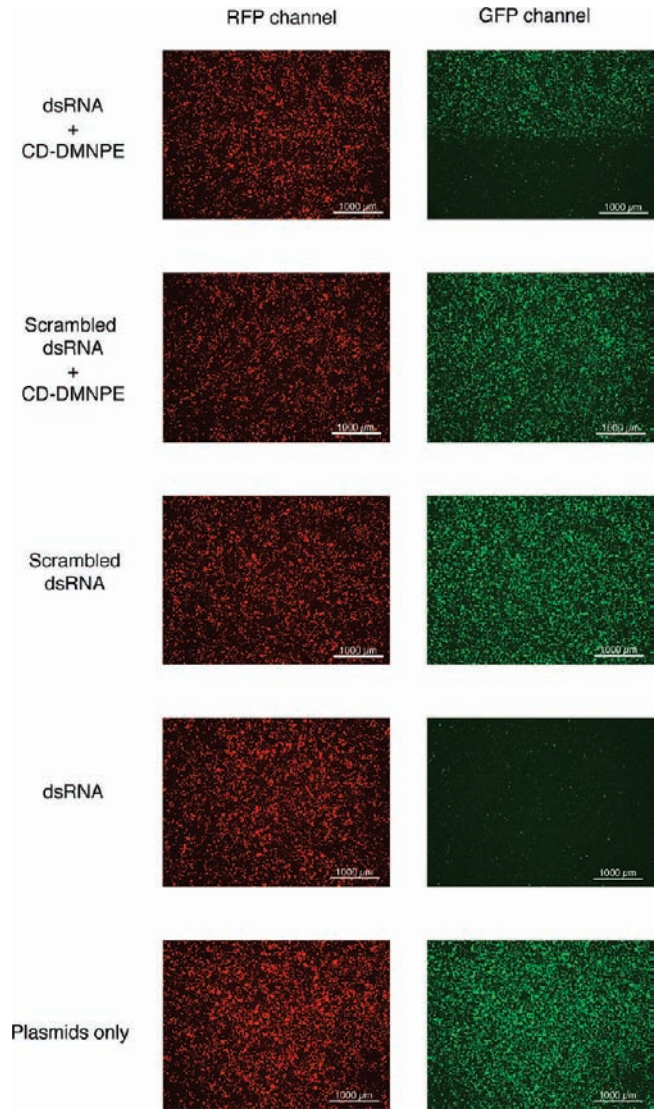

Figure 4. Patterning of gene expression using light activated RNA interference. GFP and RFP channels for each condition are indicated. Within each channel, exposure times are identical, and except for pseudocoloring to the appropriate color, no processing was done on the acquired images. A 1 mm scale bar is indicated.

also quantitated the patterning results, by reading the total GFP and RFP signals in each well. These results are shown in Figure 5. They show values consistent with the visible patterns. Most distinctively, the GFP signal for the CD-DMNPE modified GFP targeting dsRNA is slightly less than half than the GFP signal in the similarly caged scrambled sequence. If there was perfect masking, we would expect to observe exactly half the GFP signal, but we tend to observe a small amount of light leakage around the edge of our mask, resulting in GFP knockdown in areas just inside the mask border. The observed patterns of expression are completely consistent with the whole well results of Figure 3, and indicate that the CD-DMNPE group can be effectively used to pattern target gene expression with light. In addition, we have included a normalized version of Figure 5 in the Supporting Information.

To create more detailed patterns, we built a point source for irradiation, based on a high powered 200 mW, 360 nm LED (Nichia America). This source illuminates from an approximate point, which produces a sharper delineation of mask features, such as edges. The LED used has a surface of ~1 mm square,

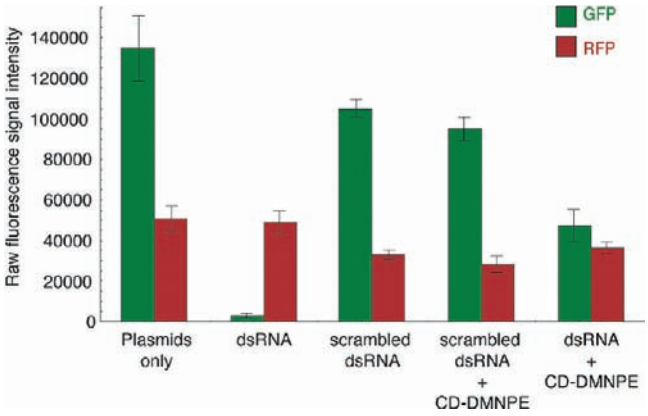

Figure 5. Quantified results of patterning experiment. Five replicates of each experimental condition were analyzed for GFP and RFP expression. The average and standard error is indicated.

and approximate isotropic intensity (90−100% of maximum intensity at angles of ≤35° of the normal). We empirically determined that we needed to irradiate for twice the duration (20 min) to produce a knockdown similar to that observed with the fluorescent bulb source. Using the point source, we produced two types of patterns: gradients of expression, and letters. Representative images of masks, and all mask image files have been included in Supporting Information.

A gradient of GFP expression was produced by using a linear gradient mask designed in Photoshop and printed on transparency film. This mask was affixed to individual wells on the underside of a 96-well culture plate. HeLa cells were plated and transfected with GFP/RFP plasmids, as well as CD-DMNPE modified, GFP targeting dsRNA, as described for previous experiments. As before, the well was irradiated 6 h after transfection and allowed to culture for 42 additional hours. The resultant pattern in both the GFP and RFP channels was captured via epifluorescent microscopy and is shown in Figure 6 (top and middle). In addition, the intensity as a function of distance across the image was quantified in both the GFP and RFP channels using Photoshop. The GFP signal normalized to the RFP signal as a function of distance is shown in Figure 6 (bottom) and shows a distinct linear gradient.

Finally, we attempted to generate more complex patterns of GFP expression, specifically letters, as a test of the limits of the approach. Letter masks were laser printed onto transparency film and affixed to the underside of 96-well plate wells, a single letter per well. As before, HeLa cells were plated and transfected with GFP/RFP plasmids, as well as CD-DMNPE modified, GFP targeting dsRNA. After 6 h of transfection, the medium was replaced and cells were irradiated using the point source. Following 40 h of culturing, the patterns of expression were visualized, using fluorescence microscopy. Representative examples of this are shown in Figure 7 combined with the RFP channel information from the same frame. They clearly show the detailed pattern of GFP expression within the frame while the untargeted RFP signal is uniform.

Discussion and Conclusion

Our aim in the execution of this work is to create a method that allows for patterns of gene expression to be generated in cells and tissues. In the past, using the classic DMNPE group, we have been limited by the window of expression that could be varied using light activated RNA interference. By incorporating the new photocleavable group cyclo-dodecyl-DMNPE into

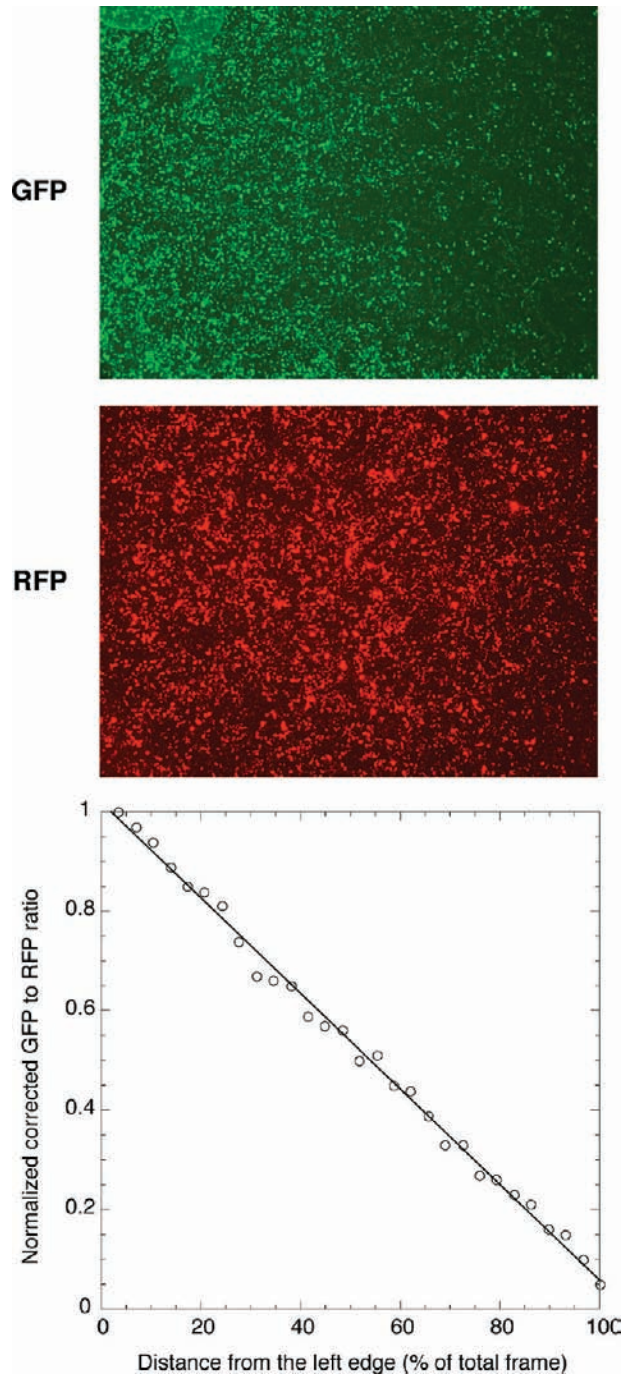

Figure 6. Gradient patterning of GFP expression using light activated RNA interference. Top 2 panels indicate GFP (target) and RFP (control) signals. Bottom panel indicates the quantification of the GFP signal normalized to RFP signal as a function of distance across the frame.

the termini of dsRNA, we have created a modified duplex that is completely blocked from RNA interference prior to irradiation, but is capable of native like knockdown after irradiation. Using these modified duplexes, we have generated robust patterns of gene expression in cell monolayers, including gradients of expression and detailed patterns.

Light activated RNA interference using CD-DMNPE has appealing features that suggest its potential for dissecting a range of biological phenomena. Because it utilizes siRNA/dsRNA, appropriate sequences for targeting any endogenous gene can be rapidly identified. By using the inherent regiospecificity of

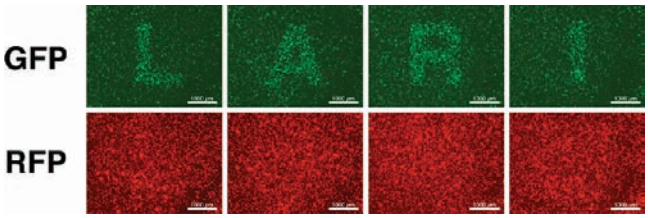

Figure 7. Letter patterning of gene expression using light activated RNA interference. GFP signal (target) and RFP signal (control) indicated. Within each channel, exposure times are identical, and except for pseudocoloring to the appropriate color, no processing was done on the acquired images. A 1 mm scale bar is indicated.

the diazo group, a regiospecificity first identified and described by our group, standard RNA with four terminal phosphates can be directly modified with four CD-DMNPE groups, by using a single reagent, diazo-CD-DMNPE.[10] This eliminates the need for extensive synthetic involvement in the creation of individual photocaged species. The resultant species allows for a complete triggering of gene expression reduction with light. This should allow for a deeper understanding of the role of gene expression on diverse biological processes including development and neuroplasticity.

Acknowledgment. We thank Prof. William Gutheil for curating the mass spectrometer used in this work. We thank Dr. Dananjay Pal and Prof. Ashim K. Mitra for access to cell culture facilities. We thank Prof. Sarah Dallas and Dr. Mark Dallas for access to the microscopy facility used in this work. The support of the University of Missouri Research Board is gratefully acknowledged.

Supporting Information Available: This includes synthetic characterization, additional replicates of patterning data described in text, and MTT toxicity results. This material is available free of charge via the Internet at http://pubs.acs.org.

JA107226E