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Photocaged Morpholino Oligomers for the Light-Regulation of Gene Function in Zebrafish and *Xenopus* Embryos

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Abstract: Morpholino oligonucleotides, or morpholinos, have emerged as powerful antisense reagents for evaluating gene function in both *in vitro* and *in vivo* contexts. However, the constitutive activity of these reagents limits their utility for applications that require spatiotemporal control, such as tissue-specific gene disruptions in embryos. Here we report a novel and efficient synthetic route for incorporating photocaged monomeric building blocks directly into morpholino oligomers and demonstrate the utility of these caged morpholinos in the light-activated control of gene function in both cell culture and living embryos. We demonstrate that a caged morpholino that targets enhanced green fluorescent protein (EGFP) disrupts EGFP production only after exposure to UV light in both transfected cells and living zebrafish (*Danio rerio*) and *Xenopus* frog embryos. Finally, we show that a caged morpholino targeting *chordin*, a zebrafish gene that yields a distinct phenotype when functionally disrupted by conventional morpholinos, elicits a *chordin* phenotype in a UV-dependent manner. Our results suggest that photocaged morpholinos are readily synthesized and highly efficacious tools for light-activated spatiotemporal control of gene expression in multiple contexts.

Introduction

Morpholino oligonucleotides were first described in 1997, as novel RNase H-resistant antisense reagents applicable to cell culture studies,[1,2] and since have been used *in vivo* to block translation,[3] modify mRNA splicing,[4] and block microRNA function.[5] Morpholinos also have become important for investigating gene function during embryonic development in species as diverse as zebrafish, medaka, carp, chicken, *Xenopus*, sea lamprey, sea urchin, and *Drosophila*.[3,4,6−9] However, these reagents must be injected directly into the cells of an early embryo and are constitutively active. Thus, morpholinos that produce early embryonic lethality eliminate the possibility of assessing gene function at later stages of development. Moreover, because the morpholino is distributed to the progeny of injected cells, spatial control of gene silencing cannot be obtained with standard antisense agents.

One strategy to eliminate the early lethality phenotypes and to achieve spatiotemporal control over gene silencing is through the application of photoresponsive morpholinos which are inactive until exposed to a short dose of non-damaging ultraviolet (UV) light.[10,11] This strategy is especially relevant to studies employing transparent zebrafish embryos: the transparent nature of the zebrafish embryo permits small regions of the embryo and even single cells to be UV-irradiated *in vivo*. A first generation of light-activated antisense agents for zebrafish embryos has been reported.[12,13] Indeed, it was recently shown that a morpholino can be linked to a short, complementary blocking morpholino via a photosensitive linker: once irradiated within a zebrafish embryo, the link between the morpholinos is broken, and the small number of base pairs is insufficient to maintain a morpholino duplex, releasing the antisense morpholino, which can then base-pair with its target mRNA.[10]

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Although effective, this strategy requires the synthesis of two morpholinos, the synthesis of the photosensitive linker, and the chemical connection of all three through covalent bond formation followed by purification.[14] The length of the linker and the sequence and length of the inhibiting morpholino need to be carefully designed. Moreover, two morpholinos are released after irradiation, which leads to a greater risk of off-target effects.[4]

An alternative strategy has been developed that utilizes a RNA-based inhibitor strand that is fully complementary to the morpholino and contains a photocleavable linkage: once irradiated within a zebrafish embryo, the link joining two short RNA oligomers is broken, and, as with the preceding strategy, the small number of RNA base pairs is insufficient for maintaining a duplex with the morpholino.[11] However, this approach requires a 5−10-fold molar excess of the inhibitor strand to saturate the morpholino, and since the injection of high amounts of nucleic acids into an embryo can be lethal, this strategy is not feasible for morpholinos that are only effective at high concentration. Furthermore, after UV irradiation, this strategy releases two RNA oligomers into an embryo, which may induce off-target effects.

Here, we present a different, substantially more *direct* approach to the generation of light-activatable morpholinos through the incorporation of a photocaged monomer during oligomer polymerization. The direct installation of light-removable (caging) groups on specific morpholino bases efficiently blocks their ability to hybridize to their target mRNA until the caging group is removed by irradiation with UV light of 365 nm. The effectiveness of this caging strategy is fully demonstrated by the UV-dependent activity of these reagents in cell culture and live embryos.

Experimental Section

General Information. Chromatography was carried out on Merck silica gel, 60 Å. NMR spectra were obtained on a Varian Oxford 300 MHz spectrometer. Absorbance spectra were obtained on an HP UV/vis spectrometer 8453. HPLC and HRMS were obtained using an Agilent Technologies 6210 LC-TOF ESI instrument.

[6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-tritylmorpholin-2-yl]methyl Acetate (2). Ac_2O (232 mg, 215 μL, 2.28 mmol) was added dropwise over 5 min to a solution of compound **1** (1 g, 2.07 mmol; gift from Gene Tools, LLC) and DMAP (spatula tip) in pyridine (10 mL) under a N_2 atmosphere at 0 °C. The reaction was allowed to stir for 12 h at room temperature. The pyridine was evaporated under vacuum, and the residue was purified by silica gel chromatography using hexanes:EtOAc (1:1) containing 1% TEA, affording **2** as a white foam in 95% yield (1.03 g, 1.97 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.38−1.48 (m, 2 H), 1.84 (s, 3 H), 2.04 (s, 3 H), 3.09−3.14 (m, 1 H), 3.35−3.39 (m, 1 H), 4.03−4.06 (m, 2 H), 4.38−4.41 (m, 1 H), 6.14−6.18 (m, 1 H), 6.99 (s, 1 H), 7.17−7.49 (m, 15 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.7, 21.0, 38.4, 49.3, 52.1, 64.7, 74.9, 76.3, 80.6, 110.9, 126.8, 128.2, 129.4, 135.6, 150.2, 164.0, 171.0. HRMS: *m*/*z* calcd for $C_{31}H_{31}N_3O_5$ $[M + H]^+$, 526.23367; found, 526.27330.

[6-(5-Methyl-3-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy]methyl}-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-tritylmorpholin-2-yl]-methyl Acetate (4). NPOM-Cl **3** (609 mg, 2.35 mmol)[29] in DMF (1 mL) was added dropwise over 5 min to a solution of compound **2** (1 g, 1.96 mmol) and Cs_2CO_3 (1.92 g, 5.88 mmol) in DMF (10 mL) under a N_2 atmosphere at 0 °C. The flask was wrapped in aluminum foil, and the reaction was allowed to stir for 12 h at room temperature. The reaction was taken up in EtOAc (100 mL) and washed with $NaHCO_3$, water, and brine (100 mL each). The organic layer was dried over Na_2SO_4 and filtered, and the volatiles were evaporated. The product was purified by silica gel chromatography using hexanes:EtOAc (2:1) containing 1% TEA, affording **4** as a yellow foam in 78% yield (1.14 g, 1.52 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.38−1.48 (m, 2 H), 1.52 (d, J = 7.2 Hz, 3 H), 1.78 (s, 3 H), 2.06 (s, 3 H), 3.12 (d, J = 11.7 Hz, 1 H), 3.35−3.39 (m, 1 H), 4.03−4.05 (m, 2 H), 4.38−4.41 (m, 1 H), 5.12−5.38 (m, 3 H), 5.95−6.13 (m, 3 H), 6.82−6.88 (m, 1 H), 7.15−7.49 (m, 17 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.4, 21.2, 24.0, 49.5, 52.6, 64.8, 70.0, 73.2, 75.0, 77.1, 81.2, 103.1, 105.0, 105.3, 106.7, 110.1, 126.7, 128.1, 129.3, 134.2, 137.9, 142.3, 146.9, 150.3, 152.2, 163.0, 170.8. HRMS: *m*/*z* calcd for $C_{41}H_{40}N_4O_{10}$ $[M + H]^+$, 749.28174; found, 749.28162. UV/vis (CH_2Cl_2): λ_{max} (ϵ) = 239 (20 000), 271 (10 000), 344 nm (4400).

1-[6-(Hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methyl-3-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy]methyl}pyrimidine-2,4(1*H*,3*H*)-dione (5). Compound **4** (1 g, 1.33 mmol) and K_2CO_3 (552 mg, 4.00 mmol) were dissolved in MeOH (10 mL) at room temperature. The reaction was stirred for 3 h, after which it was filtered, and the solvent was evaporated under vacuum. The residue was purified by silica gel chromatography using hexanes:EtOAc (1:1) containing 1% TEA, affording **5** as a yellow foam in 93% yield (874 mg, 1.24 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.38−1.45 (m, 2 H), 1.48−1.52 (m, 3 H), 1.76−1.78 (d, J = 4.5 Hz, 3 H), 1.91−1.95 (m, 0.5 H), 1.99−2.07 (m, 0.5 H), 3.08 (d, J = 11.7 Hz, 1 H), 3.28 (d, J = 12.0 Hz, 1 H), 3.51−3.62 (m, 2 H), 4.22−4.38 (m, 1 H), 5.15−5.39 (m, 3 H), 5.97−6.14 (m, 3 H), 6.85 (d, J = 9.6 Hz, 1 H), 7.15−7.49 (m, 17 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.2, 23.9, 49.0, 51.9, 64.0, 70.1, 73.1, 73.6, 77.9, 81.1, 103.0, 105.0, 105.3, 106.0, 110.2, 126.7, 128.2, 129.4, 134.2, 138.0, 142.3, 146.9, 150.5, 152.1, 163.1. HRMS: *m*/*z* calcd for $C_{39}H_{38}N_4O_9$ $[M + H]^+$, 707.27118; found, 707.27130. UV/vis (CH_2Cl_2): λ_{max} (ϵ) = 240 (20 000), 267 (10 000), 344 nm (3600).

Morpholinos. The caged compound **5** was activated and incorporated into *EGFP* and *chordin* morpholino oligomers (sequences indicated in Scheme 2) by Gene Tools, LLC[15−18] (Philomath, OR). Morpholino stocks were resuspended in water, aliquoted, and stored at −20 °C.

Morpholino Melting Temperature Determination. The melting temperature (T_m) of each morpholino hybridized to its RNA complement target was determined as described previously,[19] with the following modifications. The morpholino and RNA complement (0.5 μM) were incubated in 0.15 M NaCl, 0.05 M NaH_2PO_4, pH 7.2. The samples were protected from light or irradiated at 365 nm with an UV transilluminator (3 mW/cm^2) for 10 min, heated to 100 °C for 5 min, and then cooled to 25 °C at a rate of 2 °C/min using a Cary 100 Bio UV/vis spectrometer with a temperature controller (Varian). The absorbance was recorded at 260 nm every 1 °C. The T_m was determined by the maximum of the first derivative of the absorbance vs temperature plot. Standard deviations were calculated from three individual experiments.

Cell Culture. COS-7 cells were grown to 50−70% confluency at 37 °C and 5% CO_2 in complete culture media (DMEM supplemented with 10% FBS, 50 IU/mL penicillin, and 50 μg/mL

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streptomycin) and split into aliquots containing 10^6 cells, which were pelleted and resuspended in 100 μL of Cell Line Nucleofector Solution "R" (Amaxa Inc., Gaithersburg, MD) supplemented with 0.10 μg of pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA), 0.42 μg of pDsRed2-N1 (Clontech Laboratories, Inc.), and 150 μM morpholino (*EGFP*-MO^0 or *EGFP*-MO^4). Cell suspensions were then transfected with a Nucleofector I using program "W-01". Solutions were incubated at 25 °C for 10 min prior to the addition of 500 μL of complete culture medium. A fraction of each cell suspension (100 μL) was then transferred into a single well of a six-well plate containing 2.9 mL of complete culture medium. Plates with transfected cells were incubated at 37 °C, 5% CO_2 for 1 h prior to UV exposure. UV exposure was performed for 2 min using a DAPI fluorescence filter (340−380 nm excitation) on a Leica DM5000B compound microscope.[20] Fluorescence from transfected cells was imaged 24 h after UV exposure with a Zeiss SteREO Lumar.V12 microscope. Fluorescence images were taken at identical exposure times for control (no morpholino), no UV, and UV-exposed cells in the same experiment.

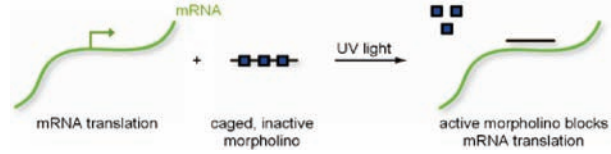
Zebrafish. All experiments involving live zebrafish (*Danio rerio*) were performed in accordance with relevant institutional and national guidelines and regulations and were approved by the North Carolina State University Institutional Animal Care and Use Committee. Zebrafish embryos were collected by natural mating, visualized with a Nikon SMZ800 microscope equipped with a 400 nm long-pass filter (GG400, Chroma Technology Corp., Rockingham, VT), and microinjected at the 1−4-cell stage with morpholinos and/or synthetic mRNAs using a pressure microinjector (PV830 picopump, World Precision Instruments, Sarasota, FL) at 60 psi and a mechanical micromanipulator (World Precision Instruments) with pulled glass capillary needles (World Precision Instruments). Embryos were photographed with a Leica DM5000B microscope equipped with a Retiga 1300 digital CCD camera and SimplePCI software.

For *EGFP* morpholino studies, zebrafish embryos were microinjected with *in vitro* transcribed capped synthetic *EGFP* mRNA (1.5 fmol, 0.40 ng), synthetic *mCherry* mRNA (0.7 fmol, 0.16 ng), and *EGFP*-MO^4 (30 fmol, 0.54 ng). All injections were made into the cytoplasm during the 1-cell stage, and some embryos were exposed to 365 nm UV light (2 min, 25 W hand-held UV lamp, 2.1 mW/cm^2) immediately following microinjection.[21] Embryos were maintained in egg water[22] with 0.5 ppm methylene blue at 28 °C for 24 h before being photographed. A 1:20 molar ratio of *EGFP* mRNA to *EGFP*-MO^4 allowed for the most efficient photoregulation of *EGFP*-MO^4 activity. Fluorescence images were taken at identical exposure times for all embryos in the same experiment.

For *chordin* studies, approximately 1 nL of a 500 μM *chordin*-MO^4, 0.15% phenol red solution was microinjected into the yolk of 1−4-cell stage zebrafish embryos. Embryos were either protected from light or exposed to 365 nm UV light (25 W hand-held UV lamp, 2.1 mW/cm^2) for 2 min at various time points after injection. Embryos were maintained in egg water[22] with 0.5 ppm methylene blue at 28 °C for 24−28 h before being scored and photographed.

Xenopus. All experiments involving live *Xenopus laevis* were performed in accordance with relevant institutional and national guidelines and regulations and were approved by the North Carolina State University Institutional Animal Care and Use Committee. *Xenopus* embryos were obtained by *in vitro* fertilization as previously described.[23] Fertilized embryos were dejellied in 2% cysteine-HCl (pH 7.8−8.1) and cultured in 0.1X Marc's Modified Ringers (MMR).[23] Before injection, embryos were equilibrated in a solution of 3% Ficoll-400 in 0.75X MMR. In each experiment, all embryos were obtained from a single clutch (i.e., eggs were from the same adult female frog). A mixture of *in vitro* transcribed capped synthetic *EGFP* mRNA (1.6 fmol, 0.42 ng), synthetic *mCherry* mRNA (7.0 fmol, 1.6 ng), and *EGFP*-MO^4 (46.8 fmol, 0.84 ng) was injected in a total volume of 0.6 nL in one animal pole blastomere at the 8−16-cell stage using a pressure microinjector (World Precision Instruments) at 60 psi and a mechanical micromanipulator (World Precision Instruments) with pulled glass capillary needles (borosilicate with rod, World Precision Instruments). A 1:30 molar ratio of *EGFP* mRNA to *EGFP*-MO^4 allowed for the most efficient photoregulation of *EGFP*-MO^4 activity. Injected embryos were recovered in the dark in 1.5% Ficoll in 0.5X MMR for 20−30 min at room temperature before being gradually transferred to 0.1X MMR. Embryos were either protected from light or exposed to UV light for 2 min at 25 °C at the 32−64-cell stage, using a Zeiss Lumar fluorescent Stereomicroscope equipped with a DAPI filter and a 1.2× objective at 120× magnification, focusing on the animal pole. Control and UV-irradiated embryos were then cultured at 16 °C until stage 24.[24] Photographs were taken using a Zeiss Lumar fluorescent stereomicroscope with an AxioCam MRc camera and Axiovision image capture software. Fluorescence images were taken at identical exposure times for all embryos in the same experiment.

Scheme 1. Photochemical Gene Regulation with Caged Morpholinos[a]



[a] Squares represent the 6-nitropiperonyloxymethyl (NPOM) caging group.

Results and Discussion

Development of NPOM-Caged Morpholinos. We hypothesized that the incorporation of a photosensitive group directly on a morpholino base would block the ability of a morpholino to base-pair with its target mRNA until the caging group is removed by UV irradiation (Scheme 1). By installing the caging group directly on the base of a phosphoramidite morpholino building block, it can be included in the direct synthesis of a morpholino and site-specifically incorporated into the oligomer, providing an efficient strategy for synthesizing caged morpholinos. This strategy is based upon earlier successes in using a novel 6-nitropiperonyloxymethyl caging group (NPOM) to disrupt Watson−Crick base-pairing in DNA:DNA and DNA:RNA duplexes.[25−28] This caging group is particularly effective at caging nitrogen heterocycles and has the advantage of disrupting hydrogen-bonding between nucleotides while providing the stability needed for use in conventional DNA or

(20) UV exposure of 1 min was not as effective at reducing EGFP expression in transfected cells.
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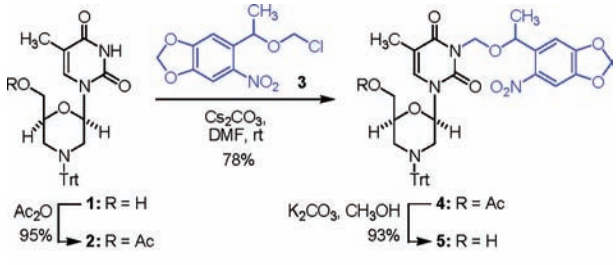
Scheme 2. Synthesis of the Caged Morpholino Monomer **5** and Synthesized Morpholino Oligomer Sequences[a]



Morpholino	Sequence
EGFP-MO^0	ACAGCTCCTCGCCCTTGCTCACCAT
EGFP-MO^4	ACAGCT*CCT*CGCCCT*TGCT*CACCAT
chordin-MO^0	ATCCACAGCAGCCCCTCCATCATCC
chordin-MO^4	AT*CCACAGCAGCCCCT*CCAT*CAT*CC

[a] T* denotes the caged monomer **5**. The NPOM caging group is shown in blue.

morpholino syntheses.[26] While stable under physiological conditions, oligonucleotides harboring these caging groups are returned to nearly full activity following a brief irradiation with 365 nm UV light.[25–28] We adopted this caging approach for the synthesis of the caged morpholino monomer **5** (Scheme 2). Compound **1** (gift from Gene Tools, LLC) was acetylated in 95% yield to deliver **2**. The NPOM caging group was then installed on **2** using the chloromethyl ether **3** (synthesized in three steps[29]) and Cs_2CO_3 in DMF at room temperature, providing **4** in 78% yield. The free 5′-hydroxyl group in **5** was obtained through saponification of the acetyl ester with K_2CO_3 in methanol (93% yield). Using standard polymerization chemistry,[16,30] the caged morpholino monomer **5** was then incorporated into morpholino sequences shown to block the translation of EGFP[31] and *chordin*[3] (Scheme 2). The morpholino sequence selected to target EGFP expression (*EGFP*-MO^0) has been shown to effectively knock down EGFP expression both in cell culture and *in vivo*.[31] The morpholino sequence selected to target the expression of endogenous zebrafish *chordin* (*chordin*-MO^0) routinely induces a specific morphological phenotype when injected into zebrafish embryos.[3,32,33] Four caged monomers were included in each morpholino, since a common control for morpholino experiments is to include a nonactive morpholino with four mismatched bases,[1,3,34] and we have shown that three to four NPOM caging groups can functionally disrupt DNA:DNA and phosphorothioate DNA:RNA duplexes.[27,28] A morpholino nomenclature was adopted in which a superscript numeral represents the number of caging groups within a morpholino (e.g., *EGFP*-MO^4 includes four caging groups, and *EGFP*-MO^0 includes zero caging groups).

NPOM Caging Groups Disrupt Morpholino:RNA Duplexes. In order to evaluate the effectiveness of NPOM caging groups to disrupt morpholinos' base-pairing, we determined the T_m for morpholino:RNA duplexes before and after 365 nm UV exposure. We evaluated *EGFP*-MO^0, *chordin*-MO^0, *EGFP*-MO^4, and *chordin*-MO^4. As indicated in Table 1 (and Supporting Information, Figure S1), the presence of four NPOM caging groups on *EGFP*-MO^4 resulted in a ~30 °C decrease in its T_m as compared to the noncaged *EGFP*-MO^0. This observation is consistent with a 27 °C decrease in T_m when four mismatches are included in a 25mer morpholino:RNA duplex.[1] Exposure of *EGFP*-MO^4 to UV resulted in increasing its T_m to be virtually the same as that of *EGFP*-MO^0. Similar experiments demonstrate that the presence of four caging groups on *chordin*-MO^4 decreases the T_m of its morpholino:RNA duplex by ~10 °C and that exposure of *chordin*-MO^4 to UV results in increasing its T_m to be nearly identical to that of *chordin*-MO^0. This smaller change in T_m is likely because the caging groups are clustered near the ends of the *chordin*-MO^4, leaving more contiguous noncaged internal bases that might weakly base-pair with its target RNA. However, the number of contiguous hybridizing bases in *chordin*-MO^4 is fewer than the reported number required for a morpholino to block gene function.[35] In addition, gel shift experiments clearly demonstrate that the NPOM caging groups on both *EGFP*-MO^4 and *chordin*-MO^4 effectively disrupt the stable formation of morpholino:RNA duplexes (Supporting Information, Figures S2 and S3); morpholino:RNA duplexes are restored with as little as a 2 min exposure to UV (Supporting Information, Figure S4). It must be noted that changes in UV source, morpholino concentration, and biological context may alter the efficiency of decaging.

Table 1. Morpholino Melting Temperatures (T_m)[a]

morpholino	irradiation	T_m (°C)
EGFP-MO^0	−UV	82.4 ± 1.0
EGFP-MO^4	−UV	53.8 ± 1.1
EGFP-MO^4	+UV	84.5 ± 1.0
chordin-MO^0	−UV	80.1 ± 0.6
chordin-MO^4	−UV	70.8 ± 0.5
chordin-MO^4	+UV	79.9 ± 1.1

[a] Experimentally determined melting temperatures (T_m) of duplexes of noncaged and caged morpholinos with their targeted RNA sequences. The distribution of the four caging groups has a substantial influence on the melting temperature of the morpholino:RNA duplexes, as discovered earlier for DNA:DNA duplexes.[27] When the caging groups are evenly distributed along the length of the morpholino (e.g., *EGFP*-MO^4), a greater change in T_m is observed. Standard deviations were determined from three independent experiments.

Spatial Control of *EGFP*-MO^4 in Transfected Cells. EGFP is an easily visualized protein and thus a highly useful target for evaluating antisense agents.[3,11,31] To assess the photosensitivity of *EGFP*-MO^4 in cells, COS-7 cells (a monkey kidney cell line) were co-transfected with a plasmid encoding EGFP, a plasmid encoding a control red fluorescent reporter (DsRed2), and either *EGFP*-MO^0 or *EGFP*-MO^4. A small region of the cell culture plate was then briefly irradiated with UV light. The *EGFP*-MO^0 effectively blocks EGFP translation, whereas *EGFP*-MO^4 blocks EGFP translation only in the region which had been exposed to UV light (Figure 1). This loss of EGFP is not the result of UV quenching, since the cells were exposed to UV light immediately after transfection, prior to EGFP production. Neither *EGFP*-MO^0 nor uncaged *EGFP*-MO^4 resulted in a complete knock-down of EGFP fluorescence in transfected cells; this is likely the result of the high levels of *EGFP* mRNA generated from the expression plasmid and/or relatively inef-

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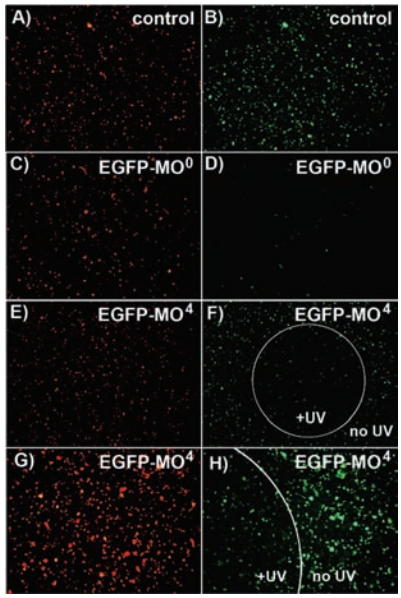

Figure 1. Spatial control of *EGFP*-MO^4 activity in mammalian cell culture. In a 96-well plate, COS-7 cells were co-transfected with plasmids encoding EGFP (pEGFP-N1) and DsRed2 (pDsRed2-N1) (A,B) with no morpholino, (C,D) with *EGFP*-MO^0, or (E,F) with *EGFP*-MO^4. Cells transfected with *EGFP*-MO^4 were briefly irradiated in a small circle at the center of the well. Expression levels of EGFP and DsRed were evaluated 24 h after transfection and irradiation, and representative images are shown on the right and left, respectively. (G,H) A magnified view of (E,F) is shown.

ficient transfection of morpholinos. Nevertheless, this experiment demonstrates that, in agreement with previous observations regarding disruption of oligonucleotide duplex formation[25–28] and our T_m and gel-shift experiments, the installation of four NPOM caging groups on a morpholino oligomer effectively inhibits antisense activity in cultured mammalian cells and that this activity can be readily restored using UV irradiation, even in a spatially controlled fashion.

***EGFP*-MO^4 Regulation of EGFP Expression in Zebrafish Embryos Is UV-Dependent.** We then assessed whether the caged *EGFP*-MO^4 is effective in regulating gene expression *in vivo*. We utilized the zebrafish embryo, a developmental model organism with transparent embryos in which morpholinos are routinely employed.[3,34] Embryos were co-injected at the 1−4-cell stage with *in vitro* transcribed, synthetic mRNA encoding EGFP, *EGFP*-MO^4 and synthetic mRNA encoding the red fluorescent protein mCherry (as a control). Figure 2 demonstrates that, in the absence of UV irradiation, injected embryos express both EGFP and mCherry and that UV exposure specifically disrupts EGFP expression. Importantly, zebrafish embryos exposed to UV light display a normal phenotype (also see Figure 4F, below).[36] As expected, embryos co-injected with *EGFP* mRNA and *EGFP*-MO^0 display little or no EGFP expression, regardless of UV exposure (data not shown). In addition, this loss of EGFP expression is not the result of UV quenching, as the embryos are exposed to UV light immediately after injection, well prior to EGFP protein production. These results demonstrate that NPOM-caged morpholinos can be used to achieve effective light-controlled regulation of gene expression in live embryos.

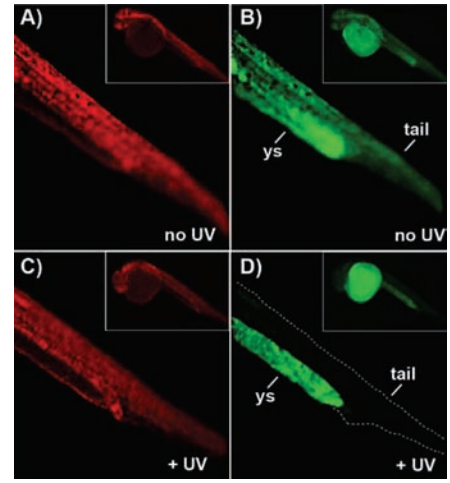

Figure 2. Light-activation of *EGFP*-MO^4 in zebrafish embryos. Zebrafish embryos at the 1−4-cell stage were microinjected with synthetic mRNA encoding EGFP, *EGFP*-MO^4 (1:20 molar ratio), and synthetic mRNA encoding mCherry and were (A,B) protected from light or (C,D) immediately exposed to UV light. Expression levels of EGFP and mCherry were evaluated 24 h after injection and irradiation, and representative images of zebrafish tails are shown on the right and left, respectively. The fluorescence observed in (D) is not derived from EGFP but is autofluorescence of the yolk sac (ys), as previously observed.[37] Photos of entire embryos are shown in insets.

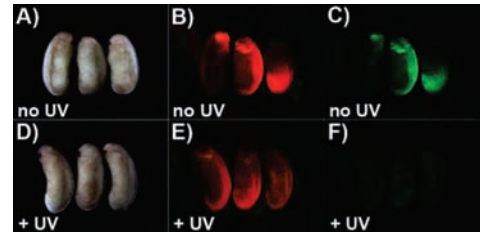

Figure 3. Light-activation of *EGFP*-MO^4 in *Xenopus* embryos. *Xenopus* embryos were microinjected with synthetic mRNA encoding EGFP, *EGFP*-MO^4 (1:30 molar ratio), and synthetic mRNA encoding mCherry and (A−C) protected from light or (D−F) immediately exposed to UV light. Expression levels of EGFP (right) and mCherry (middle) were evaluated at embryonic stage 24 (~1 day after injection) and are shown in comparison to bright-field images (left).

***EGFP*-MO^4 Regulation of EGFP Expression in *Xenopus* Embryos Is UV Dependent.** To test the broader applicability of this approach for species beyond zebrafish, the caged *EGFP* morpholino was also investigated in *Xenopus* frog embryos. Although not as optically transparent, caged compounds have successfully been used in *Xenopus*.[37,38] One advantage of *Xenopus* is the ability to target specific tissues by injecting individual cells of the early embryo to limit the distribution of injected reagents.[39] *Xenopus* embryos were co-injected in a single cell at the 8−16-cell stage with synthetic *EGFP* mRNA, *EGFP*-MO^4, and synthetic *mCherry* mRNA. All injected embryos were phenotypically normal, and Figure 3 clearly demonstrates that, although mCherry expression was unaffected, *EGFP*-MO^4 blocks EGFP production only after UV exposure. As in zebrafish, this loss of EGFP expression cannot be the result of UV quenching, as the embryos are exposed to UV light immediately after injection, which is prior to EGFP

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production. In conclusion, the direct caging approach can be used for the light-regulation of morpholino activity in cell culture, zebrafish embryos, and *Xenopus* embryos.

***chordin*-MO^4 Regulation of the *chordin* Gene in Zebrafish Embryos Is UV Dependent.** The above results show that our NPOM-caged *EGFP* morpholino enables light-regulated control of EGFP expression in cell culture and *in vivo*. In order to determine if NPOM-caged morpholinos can effectively regulate endogenous genes, we chose to target the zebrafish *chordin* gene. We selected a well-characterized morpholino sequence (*chordin*-MO^0) which, when injected into 1−4-cell zebrafish embyros, induces a shrunken head and a ventralized tail by 24−28 h post fertilization (hpf), characteristic of the *chordin* phenotype.[3,32,33] We routinely observe >90% of *chordin*-MO^0-injected embryos displaying these phenotypes (data not shown). To evaluate the light-dependent efficacy of a NPOM-caged *chordin* morpholino (*chordin*-MO^4), it was injected into 1−4-cell stage zebrafish embryos which were then either protected from light or immediately subjected to a brief exposure to UV. Embryos were allowed to develop until 24−28 hpf, at which time their phenotype was assessed, and the severity of the *chordin* phenotype was scored as normal, mild, moderate, or severe (Figure 4A−D). The moderate and severe phenotypes are deformities known to be induced by disruption of the *chordin* gene,[3,32,33] and the mild phenotype is characterized by a slight blunting in the animal's tail. We suggest that this latter phenotype is caused by a small amount of partial decaging of *chordin*-MO^4 through handling under ambient light or by the stretch of contiguous noncaged bases within *chordin*-MO^4 weakly associating with its target mRNA. Note that the mild phenotype is not caused merely by the presence of a NPOM-caged morpholino, as this phenotype is not observed when embryos are injected with *EGFP*-MO^4 (data not shown). One hundred percent of control embryos that are not injected with a morpholino displayed a normal phenotype at 24−28 hpf, independent of UV exposure, confirming that irradiation does not induce any observable developmental defect (Figure 4E,F; no UV, $n = 26$; +UV, $n = 32$). The observation that 99% of *chordin*-MO^4-injected embryos not exposed to UV displayed normal or mild phenotypes at 24−28 hpf confirms that the NPOM-caged morpholinos are stable in the zebrafish embryo (Figure 4G; $n = 74$ embryos). In contrast, 90% of *chordin*-MO^4-injected embryos that are immediately exposed to UV light displayed moderate and severe phenotypes (Figure 4H; $n = 88$ embryos), suggesting an efficient removal of the NPOM caging groups *in vivo* after UV exposure, activation of the morpholino antisense agent, and subsequent knock-down of *chordin* function.

In order to determine if NPOM-caged morpholinos can be employed for temporal control of gene expression, zebrafish embryos were injected with *chordin*-MO^4 and irradiated at successively later stages of development (Figure 4I). Interestingly, exposure of *chordin*-MO^4-injected embryos to UV at any time point prior to the developmental stage of gastrulation (~10 hpf) reliably induced a severe *chordin* mutant phenotype, while disruption of *chordin* during mid- to late-stage gastrulation elicited milder anomalies. These results are highly consistent with the known expression patterns and the timing of the dorsalizing function of *chordin* in the zebrafish embryo.[3] These results confirm that the NPOM-caged morpholino technology

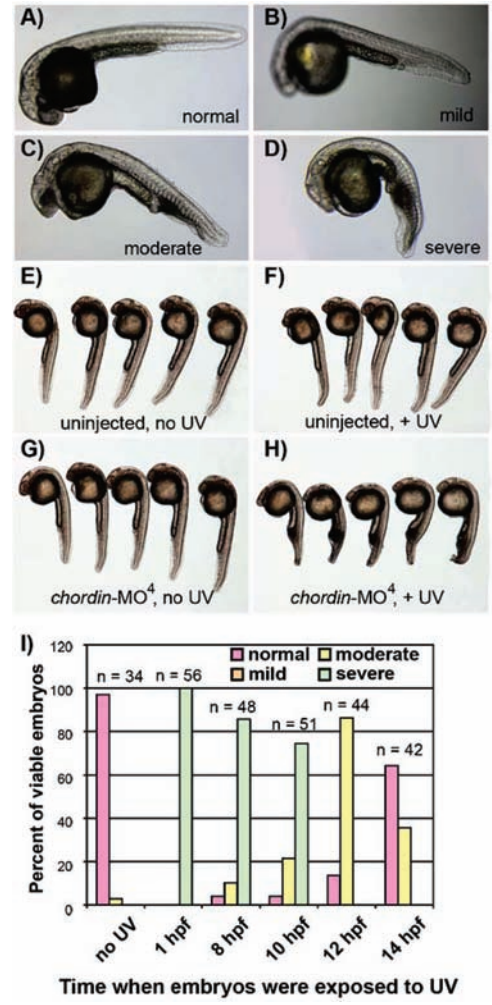

Figure 4. Light-activation of *chordin*-MO^4 in zebrafish embryos. (A−H) Zebrafish embryos were microinjected with *chordin*-MO^4 during the 1−4-cell stage and either protected from light or immediately irradiated with UV light, and their phenotype was assessed 24−28 h post fertilization (hpf). Phenotypes include (A) normal, (B) mild, (C) moderate, and (D) severe. (E,F) Uninjected control embryos display a normal phenotype regardless of UV exposure. (G) Embryos injected with *chordin*-MO^4 and not exposed to UV light of 365 nm display normal or mild phenotypes. (H) Embryos injected with *chordin*-MO^4 and immediately exposed to UV light display moderate and severe phenotypes. (I) Embryos were injected with *chordin*-MO^4 as described above and divided into groups that were irradiated at various time points after fertilization, and their phenotype was assessed at 24−28 hpf. Note that light-activation of *chordin*-MO^4 during or after the developmental stage of gastrulation (~10 hpf) fails to generate the severe *chordin* phenotype.

is applicable to the light-regulation of different genes and allows for the temporal investigation of gene function in live zebrafish embryos.

Conclusion

We have developed a caged morpholino monomer and incorporated it in the synthesis of two different morpholino antisense reagents. These caged antisense agents were inactive in cell culture, zebrafish embryos, and *Xenopus* embryos until irradiated with UV light of 365 nm. Using these reagents, we demonstrated photochemical gene silencing in mammalian cells and in live aquatic animals and observed the expected embryonic phenotypes. No detectable toxic effects of the irradiation or the caging groups were observed. The developed direct caging approach can be easily applied to any morpholino oligomer,

since the straightforward design only requires insertion of caged building blocks in the oligomer synthesis. We are currently developing the chemistry to synthesize caged morpholino monomers for the other three bases, which will provide greater flexibility in the design of caged morpholino oligonucleotides. It is likely that this methodology will find broad application in the cell and developmental biology research community, since it is generally applicable to the spatial and temporal regulation of gene function in multiple biological contexts.

Acknowledgment. We thank Gene Tools, LLC for synthesizing caged morpholino oligomers from our caged building block, Syntrix Biosystems, Inc. for fruitful discussions, and R. Read Tull for assistance with *Xenopus* microinjections. Financial support from the National Institutes of Health (R01GM079114, R01DK085300, and R43GM088964) is acknowledged. A.D. is the recipient of a Beckman Young Investigator Award and a Cottrell Scholar Award.

Supporting Information Available: Melting curves for caged morpholinos and morpholino gel shift assay results. This material is available free of charge via the Internet at http://pubs.acs.org.

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