Supporting Information

Evaluating the Mechanisms of Light-Triggered siRNA Release from Nanoshells for Temporal Control Over Gene Regulation

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Nanoshell Synthesis, Functionalization, and Characterization

Nanoshells (NS) were synthesized according to published methods.[1] First, 120-nm silica spheres functionalized with 3-aminopropyltriethoxysilane (Nanocomposix), 1 M sodium chloride, and 3-4 nm diameter gold colloid made by the Duff method[2] were combined and rocked for several days at room temperature. The resultant solution of "seed" nanoparticles was centrifuged twice and resuspended in milliQ water to an optical density (OD) of 0.1 at 530 nm. The diluted seed was then mixed with additional hydrogen tetrachloroaurate(III) hydrate ($HAuCl_4$) diluted in potassium carbonate, and following addition of formaldehyde the sample was rapidly agitated to form complete gold shells. NS were treated with 0.1% diethyl pyrocarbonate (DEPC) for 3 days at 37°C to render the solution RNase-free. All materials described were purchased or treated with DEPC to be RNase-free prior to use.

siRNA oligonucleotides were purchased as single strands from IDTDNA (sequences listed in **Table S1**). Thiolated sense strands were mixed with complementary non-thiolated antisense strands in equimolar amounts, boiled at 95°C for 5 min, and then slowly cooled to 37°C over 1 hr to facilitate siRNA duplexing. RNase-free NS were diluted to $4.1x10^9$ NS/mL (corresponding to OD=1.5) in milliQ water, and Tween-20 and 5 M NaCl were added to final levels of 0.2% and 20 mM, respectively. siRNA duplexes were added to NS to a final concentration of 200 nM, and the solution was bath sonicated and rocked at room temperature. Samples were salt aged incrementally to a final NaCl concentration of 400 mM prior to rocking overnight. Next, 5 kDa methoxy-poly(ethylene) glycol-thiol (mPEG-SH) was diluted in milliQ water to 1 mM and added to NS to a final concentration of 5 μM mPEG-SH. After rocking for 4 hr at 4°C, the NS solutions were purified by centrifugation 3 times, resuspended in 200 μL 1X RNase-free phosphate buffered saline (PBS), and stored at 4°C until use.

NS concentrations were calculated based on Beer's law using the peak extinction (~800 nm) measured on a Cary 60 UV-visible spectrophotometer. To quantify the number of siRNA loaded onto NS, 200 μL of NS diluted to $4.1x10^9$ NS/mL was combined with 200 μL urea, then this solution was heated and mixed at 45°C for 20 min. The solution was centrifuged and the supernatant collected. Sample fluorescence for Cy5-labeled siRNA was read on a Biotek Synergy H1 microplate reader and compared to a standard curve of known antisense concentration (0-20 nM). siRNA content on siGFP-NS and siSCR-NS without Cy5 labels were similarly quantified using the Quant-iT OliGreen™ ssDNA quantification kit (Thermo Fisher) according to manufacturer recommendations and as detailed previously.[3]

Characterization of siRNA Release Upon Continuous Wave (CW) or Pulsed Laser Irradiation

siRNA release characterization was performed using NS functionalized with the scramble GFP sequence tagged with a Cy5 fluorophore on the 3′ end of the antisense strand. Cy5-siRNA-NS were diluted in RNase-free milliQ water to $5.5x10^9$ NS/mL (corresponding to OD=2), and 2 mL was placed into RNase-free disposable cuvettes with stir bars and samples stored on ice until use. For both CW and pulsed laser experiments, samples were irradiated from the side while stirring (**Figure S1**). An 808 nm CW laser (B&W Tek) was applied at 0, 5, 10, 20, or 25, or 30 W/cm^2 with a 2 mm spot size (corresponding to 0, 157, 314, 628, 785, 942 mW output powers) for 30

min. Thermal images were taken every 5 min with an FLIR A5 thermal camera (FLIR Systems) and the highest temperature at each timepoint was recorded. The 800 nm pulsed laser utilized in these experiments (Coherent) was equipped with a modelocked Ti:Sapphire oscillator (Mantis) and a regenerative amplifier (Legend Elite). Samples were irradiated with a 10 kHz repetition rate and 40 fs pulse length. Samples exposed to this laser were irradiated with a 50 μm spot size focused using a fused silica lens with a focal length of 30 cm. siRNA-NS were irradiated with 1, 2, or 3 mW output power for 10, 20, or 30 min.

Following irradiation with the CW or pulsed laser, samples were read on a Cary60 UV-vis spectrophotometer, centrifuged to form a pellet (1500x*g*, 5 min), and the supernatant containing any released RNA was collected. Sample Cy5 fluorescence was read on the Synergy H1 plate reader and compared to a standard curve of known siRNA concentration (0-10 nM). The remaining supernatant was lyophilized overnight for gel electrophoresis. For this, samples were diluted in milliQ water and RNA content was determined using a Take3 plate on the Synergy H1 plate reader using the absorbance at 260 nm. Samples were combined with 2X formamide buffer, boiled at 99°C for 3 min, and quickly cooled on ice. 100 ng of siRNA duplexes, Cy5-antisense strands, thiolated sense strands, or released siRNA was run on 4-20% TBE gels (Lonza) at 125-175 V for 1 hr in 1X TBE buffer. Then, gels were placed into 1X SYBR Gold (Thermo Fisher) diluted in TBE buffer for 20 min. Lastly, gels were imaged using the ethidium bromide filter on a ChemiDoc-iTTM2 Imager (UVP) with a 5 sec exposure time. Band intensity was analyzed in Fiji (ImageJ) software using the gel analyzer feature.

Following centrifugation, the NS-containing pellets were suspended in 2 mL milliQ water and analyzed by dynamic light scattering for hydrodynamic diameter and by flow cytometry for Cy5 fluorescence intensity using a forward scatter cutoff of 10 and the APC filter (excitation/emission 640 nm/660 nm).

U373.eGFP Cell Culture

U373.eGFP cells were gifted from Dr. Susan Blaney of Texas Children's Hospital and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured in T75 cell culture flasks, passaged with 0.25% trypsin-EDTA, and incubated at 37°C in a 5% CO_2 environment. Flasks were grown to 80-90% confluence prior to plating for experiments.

Transfection with Released GFP siRNA

U373.eGFP cells were detached from flasks with 0.25% trypsin-EDTA, counted on a hemocytometer, plated at 5,000 cells/well in 96 well plates in complete cell culture media, and incubated overnight. siGFP-NS and siSCR-NS were diluted in RNase-free water to $5.5x10^9$ NS/mL (OD=2), and 4 mL of each sample type was placed into cuvettes with stir bars. The NS samples were irradiated with the pulsed laser at 3 mW for 30 min while stirring. Then, samples were centrifuged to form a pellet and the supernatant containing released RNA was collected and lyophilized overnight. Following lyophilization, the released siRNA was diluted in RNase-free water and the RNA concentration was determined using the Take3 plate on the Synergy H1 plate

reader using the absorbance at 260 nm. To examine whether the released RNA retained its functionality, plated U373.eGFP cells were transfected with 100 nM of the released GFP or scramble siRNA with Dharmafect (Dharmacon) per manufacturer recommendations, and after 6 hr the transfection solution was replaced with antibiotic-free media. Cellular GFP expression was analyzed at 48 hr and 96 hr post-transfection by fluorescence imaging with an EGFP filter on a Zeiss Axioobserver Z1 Inverted Fluorescence Microscope. For flow cytometry, cells were lifted with 0.25% trypsin-EDTA and treated with propidium iodide (PI, Thermo Fisher) for 5 min at room temperature per manufacturer recommendations to enable the exclusion of dead cells by flow cytometry. To analyze the flow cytometry data throughout this paper, first the cell population was selected using forward scatter versus side scatter density plots. Then, singlet cells were gated using forward scatter height versus area density plots. Finally, GFP expression was determined using the FITC channel (excitation/emission 488 nm/530 nm). The median fluorescence intensity (MFI) data shown are the averages and standard deviations from three independent experiments.

Light-Triggered GFP Silencing in U373.eGFP Cells with siRNA-NS

To assess cell uptake of siRNA-NS conjugates, U373.eGFP cells were plated at 5,000 cells/well in 96 well plates and incubated overnight. Cy5-siRNA-NS diluted $1.4x10^{10}$ NS/mL (OD=5) in complete cell culture media were added to the cells, which were then incubated for 3 hr. For flow cytometry, cells were lifted with 0.25% trypsin-EDTA, centrifuged, suspended in 1X PBS, and the Cy5 signal was measured using the APC filter. For imaging, cells were fixed with 4% formaldehyde in PBS for 15 min and then treated with DAPI overnight at 4°C to stain cell nuclei. After overnight incubation, cells were imaged on the Zeiss Axioobserver Z1 fluorescent microscope using the DsRed, EGFP, and DAPI filter sets.

For GFP knockdown studies, U373.eGFP cells were plated at 100,000 cells/well in 6-well plates and incubated overnight. Then, cells were treated with 2 mL of siGFP-NS or siSCR-NS diluted to $1.4x10^{10}$ NS/mL (OD=5) in complete cell culture media. After 3 hr, cells were detached with 0.25% trypsin-EDTA, centrifuged 1X at 200x*g* for 5 min, and suspended in 1 mL of sterile 1X PBS. Cells were placed into sterilized cuvettes with stir bars and kept on ice throughout the duration of the experiment, except during irradiation. While the NS-treated cells stirred in the cuvettes, the femtosecond pulsed laser was applied at 0 or 3 mW for 20 min. After irradiation, the cells were centrifuged, suspended in media, counted, and plated in 24 well plates at 45,000 cells/well. The media was replaced every day, and the cells were analyzed for GFP expression using fluorescence microscopy (EGFP channel) and flow cytometry (FITC channel) 4 days after light treatment. The MFI data shown are normalized to the siSCR-NS treated cells in each experiment and then averaged together from four independent experiments. Statistics were analyzed by one-way analysis of variance with posthoc Tukey Kramer.

(1) Oldenburg, S. J.; Averitt, R. D.; Westcott, S. L.; Halas, N. J. *Chem. Phys. Lett.* **1998**, *288*, 243–247.

(2) Duff, D. G.; Baiker, A.; Edwards, P. P. *ACS Langmuir* **1993**, *272* (16), 2301–2309.

(3) Melamed, J. R.; Riley, R. S.; Valcourt, D.; Billingsley, M.; Kreuzberger, N.; Day, E. S. *Quantification of siRNA duplexes bound to gold nanoparticle surfaces*, 2nd ed.; Springer, 2016.

Name	Sequence
GFP Antisense	GGUGCGCUCCUGGACGUAGCCTT
GFP Sense	GGCUACGUCCAGGAGCGCACCTT-(Sp18)(Sp18)-Thiol
Scram Antisense	UGCCGAUCGGCACGGUCGCGUTT
Scram Sense	ACGCGACCGUGCCGAUCGGCATT-(Sp18)(Sp18)-Thiol
Cy5 Scram Antisense	UGCCGAUCGGCACGGUCGCGUTT-Cy5

Table S1. siGFP, siSCR, and Cy5 siSCR sense and antisense RNA sequences used in this work, denoted 5' to 3'.

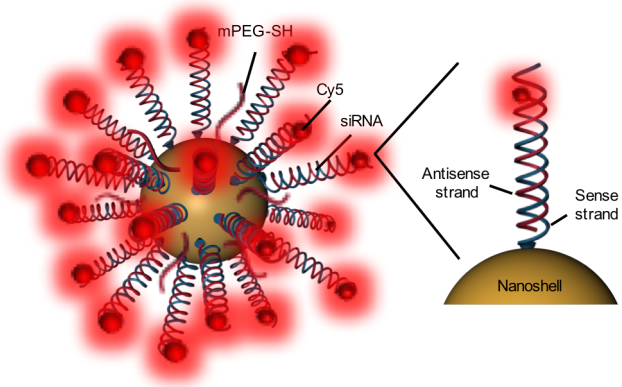

Figure S1. Schematic of Cy5-siSCR-NS showing position of Cy5 fluorophore on the 3' end of the antisense strand of the siRNA.

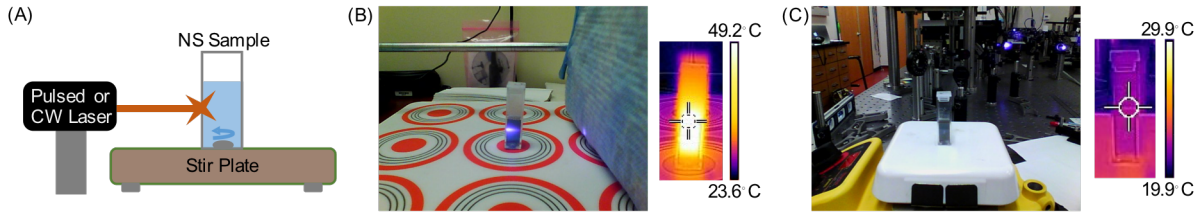

Figure S2. (A) Schematic of irradiation setup for bulk NS solutions under both CW and pulsed laser irradiation (schematic not to scale). (B) and (C) Photographs and thermal images showing the 808 nm CW laser experimental setup, and the pulsed irradiation experimental setup, respectively.

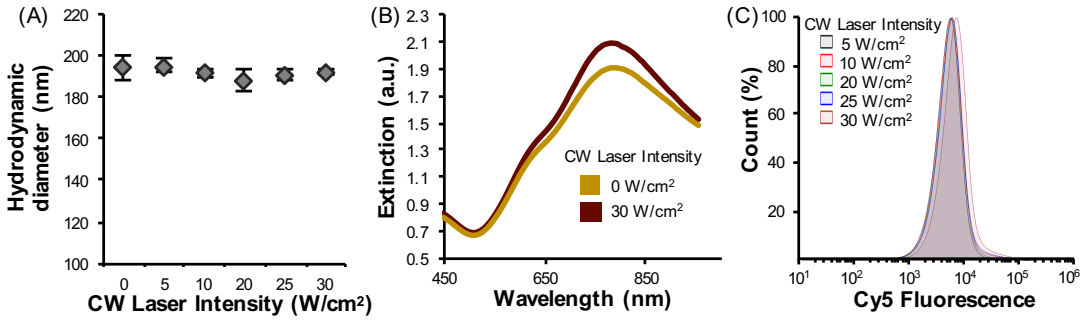

Figure S3. (A) Hydrodynamic diameter of Cy5-siSCR-NS following irradiation with the 808 nm CW laser at 0, 5, 10, 20, 25, or 30 W/cm^2 for 30 min. (B) UV-visible spectrophotometry of Cy5-siSCR-NS that were irradiated with 808 nm CW light for 30 min. (C) Flow cytometry showing Cy5 fluorescence from Cy5-siSCR-NS after CW irradiation for 30 min at 5, 10, 20, 25, or 30 W/cm^2.

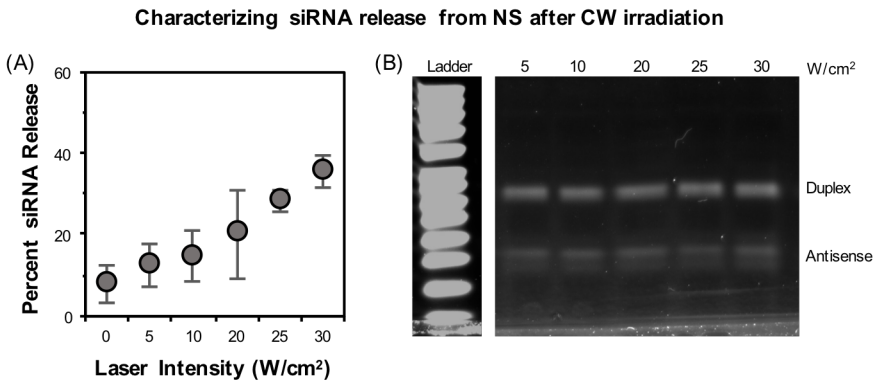

Figure S4. (A) Quantification and (B) Gel electrophoresis of siRNA released from NS following 808 nm CW irradiation at 0, 5, 10, 20, 25, or 30 W/cm^2 for 30 min.

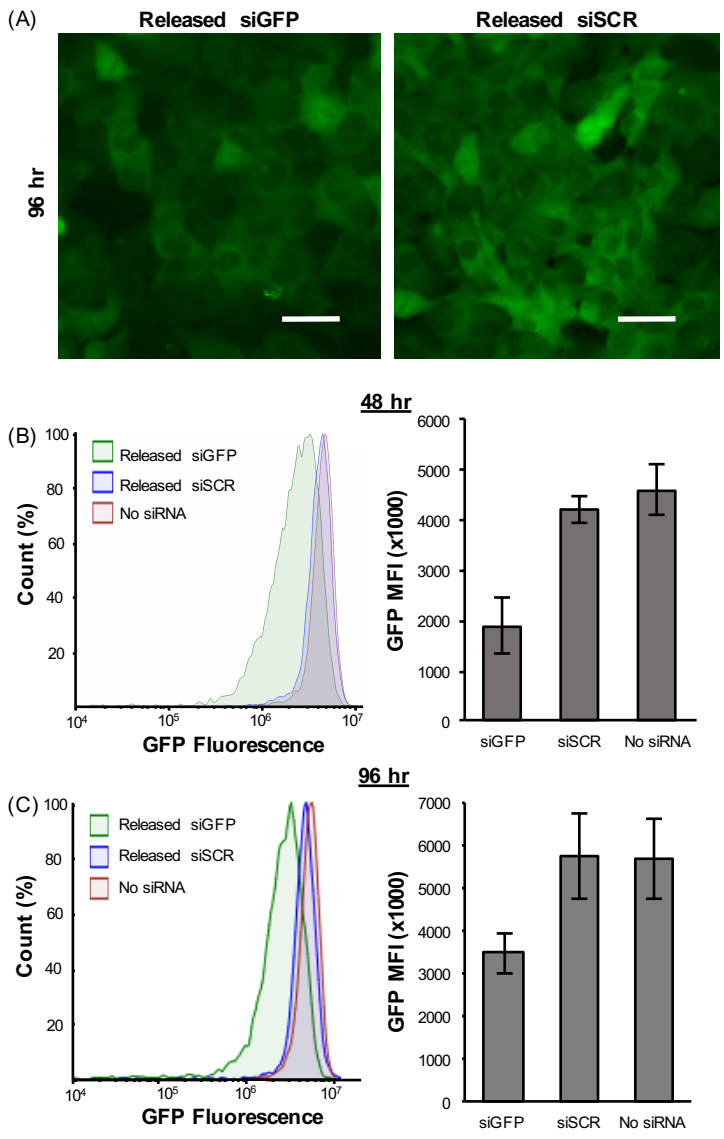

Figure S5. (A) Fluorescence images and (B and C) flow cytometry analyses showing GFP expression in U373 cells transiently transfected with 100 nM GFP siRNA, SCR siRNA, or no siRNA that was released from NS using 3 mW femtosecond pulsed laser for 30 min while stirring. The data shown is (B) 48 hr and (C) 96 hr post-transfection. Scale=50 μm.

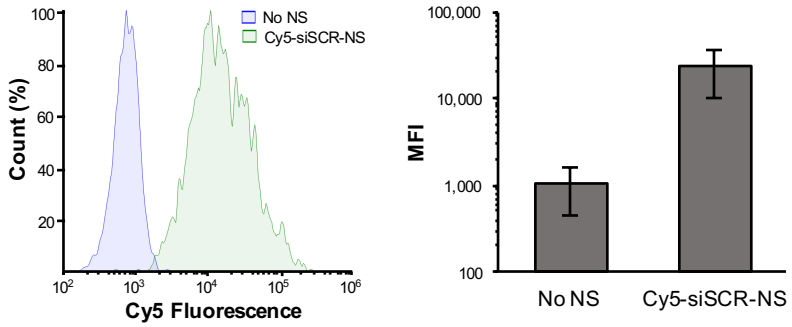

Figure S6. Flow cytometry analysis of Cy5-siSCR-NS uptake by U373.eGFP cells following a 3 hr incubation period. Control cells were treated with no NS.

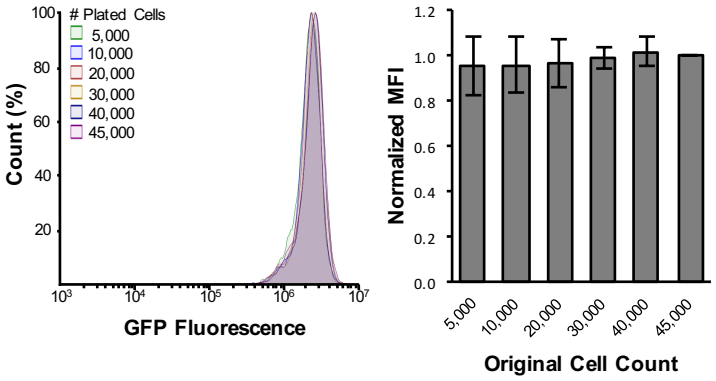

Figure S7. Flow cytometry analysis showing GFP expression does not change based on cell confluency.