

Photonic Gene Circuits by Optically Addressable siRNA-Au Nanoantennas

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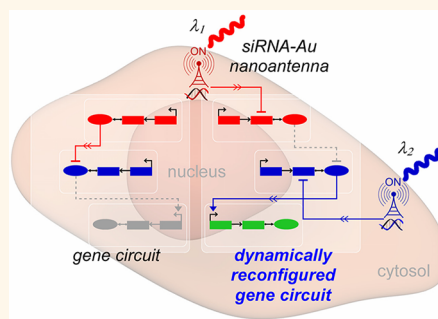
Gene circuits, consisting of interconnected genes and proteins, give rise to cell functions essential in signaling, growth, differentiation, and apoptosis. Despite some initial success, many important gene circuits remain incompletely understood. Increased understanding of information processing and operation of gene circuits should advance therapeutic strategies for reconfiguring gene circuits involved in disease progression and cancer. A major challenge is probing native gene circuits with high signal fidelity. Top-down probing approaches utilize an external input signal and essentially treat a living cell as an input–output “black box”. Ideally, the output signal should be directly correlated to the input signal. However, signal distortions—time delays, noise, and signal magnitude reductions—can confound this input–output relationship,¹ hindering temporally precise modulation of gene circuits and limiting dynamic reconfiguration of gene circuits in living cells.

Intracellular approaches that enable temporally precise modulation of native gene circuits and dynamic reconfiguration of existing circuit connections will have widespread applications in fundamental biology and translational medicine. Optical input signals are a promising interface to gene circuit connections. Current optogenetic methods to interface with living cells have so far relied on genomic modifications (*i.e.*, mutations, overexpression) to permanently encode these cells with light-responsive genes,² limiting on-demand reconfiguration of gene circuits in living cells. To enable on-demand circuit reconfiguration, remotely controlled inputs to gene circuits are required. Previously, remote control of biomolecules has been demonstrated by inductive coupling of radio frequencies to

ABSTRACT The precise perturbation of gene circuits and the direct observation of signaling pathways in living cells are essential for both fundamental biology and translational medicine. Current optogenetic technology offers a new paradigm of optical control for cells; however,

this technology relies on permanent genomic modifications with light-responsive genes, thus limiting dynamic reconfiguration of gene circuits. Here, we report precise control of perturbation and reconfiguration of gene circuits in living cells by optically addressable siRNA-Au nanoantennas. The siRNA-Au nanoantennas fulfill dual functions as selectively addressable optical receivers and biomolecular emitters of small interfering RNA (siRNA). Using siRNA-Au nanoantennas as optical inputs to existing circuit connections, photonic gene circuits are constructed in living cells. We show that photonic gene circuits are modular, enabling subcircuits to be combined on-demand. Photonic gene circuits open new avenues for engineering functional gene circuits useful for fundamental bioscience, bioengineering, and medical applications.

KEYWORDS: siRNA · RNA interference · optogenetics · plasmonics · gene delivery · gene therapy · gold nanorod



nanocrystal antennas.³ Recently, remote optical control of biomolecules, such as DNA⁴ and siRNA,⁵ has been demonstrated by coupling optical frequencies to resonant optical nanoantennas.^{4–16} By receiving and focusing freely propagating optical radiation into localized energy, resonant optical nanoantennas efficiently convert absorbed optical energy into surface-localized heat, otherwise known as photothermal effect.^{17–22} When biomolecules are functionalized to the surface of the nanoantenna, this optically generated, surface-localized heat can be used to liberate (*i.e.*, emit) biomolecules from the nanoantenna. Inside of living cells, optical silencing of genes (Figure S9) has been previously

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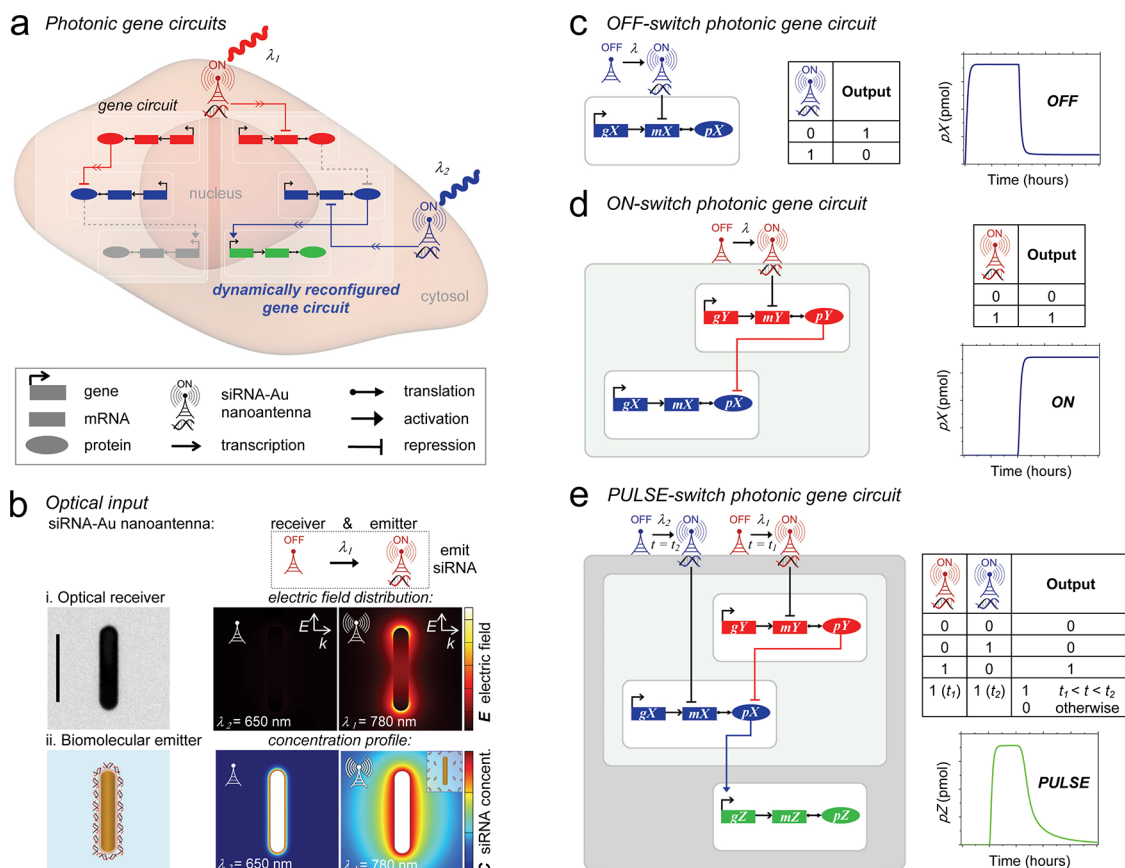


Figure 1. Photonic gene circuits. (a) Dynamic optical circuit reconfiguration is enabled by resonant siRNA-Au nanoantennas as optical inputs to existing circuit connections of living cells, forming photonic gene circuits. Legend notates symbols and circuit connections. (b) Resonant siRNA-Au nanoantennas function as selectively addressable optical receivers and biomolecular emitters of siRNA. (i) Transmission electron microscopy image of the nanoantenna structure (gold material). Scale bar is 50 nm. Electric field calculated using excitation $\lambda = 780 \text{ nm}$ or $\lambda = 650 \text{ nm}$. Antenna effect is "on" at the resonance state and "off" at the nonresonance state of the nanoantenna. (ii) Conceptual image of siRNA-Au nanoantenna. The nanoantenna surface is modified with a cationic phospholipid bilayer, and negatively charged siRNA is adsorbed to the surface, forming the siRNA-Au nanoantenna. Calculated concentration profiles: siRNA emitted from the nanoantenna when the antenna effect is "on" at the resonance state, and no siRNA emitted when the antenna effect is "off" at the nonresonance state. Inset: Conceptual image of siRNA emission from optically addressed siRNA-Au nanoantenna. (c) Circuit diagram, logic table, and model of OFF-switch photonic gene circuit. (d) Circuit diagram, logic table, and model of ON-switch photonic gene circuit. (e) Circuit diagram, logic table, and model of PULSE-switch photonic gene circuit.

demonstrated by liberating interfering oligonucleotides from nanoantennas using optically generated, surface-localized heat.^{4,7,8,12} However, the optical gene silencing mechanism has not yet been utilized, in the context of gene circuits, to dynamically reconfigure existing gene circuits and optically engineer new gene circuit configurations.

Here, on-demand optical circuit reconfiguration is enabled by optically addressable siRNA-Au nanoantennas functioning as optical receivers and biomolecular emitters of siRNA via the optical gene silencing mechanism. In this manner, siRNA-Au nanoantennas serve as optical inputs to existing circuit connections (Figure 1a), forming photonic gene circuits (Figure 1c, d, e). Photonic gene circuits are modular, allowing subcircuits to be combined on-demand. This work represents a new way to engineer functional gene circuits, as it allows, for the first time, temporally precise modulation and dynamic reconfiguration of native gene circuits.

RESULTS AND DISCUSSION

Among the different nanoantenna structures of current widespread interest, a rod-shaped Au nanoantenna (i.e., Au nanorod) was selected here (Figure 1b, i) because of its large optical absorption cross-section, narrow spectral bandwidth of the longitudinal plasmon resonance band, and tunable longitudinal plasmon resonance wavelength in the near-infrared (NIR) regime, where cells are essentially transparent.²³ The optical properties of an Au nanorod are dominated by its plasmon resonance. At resonance, the optical near-field shows two regions of high-field intensity (Figure 1b, i) due to charge accumulation at each end of the nanoantenna.²⁴ These regions of high-field intensity indicate that the nanoantenna functions as an optical receiver to focus freely propagating optical fields down to nanometer-scale dimensions.²⁵ This antenna effect is prominent at the resonance state of

the nanoantenna, where the excitation wavelength is matched with the plasmon resonance wavelength. In Figure 1b, i, this antenna effect is seen to be “on” at the resonance state and “off” at the nonresonance state of a nanoantenna, where the resonance and nonresonance states depend on aspect ratio. We exploited this antenna effect to design nanoantennas, based on aspect ratios, that can be optically addressed with minimal antenna crosstalk for use in constructing photonic gene circuits.

For use as biomolecular emitters, resonant siRNA-Au nanoantennas were designed such that the photo-thermal heat transfer from the nanoantenna surface to the surrounding cellular environment is highly localized, decaying exponentially,²⁶ and therefore is thought to have minimal adverse effects on cells. To further minimize adverse effects, the plasmon resonance wavelength is also tuned to the NIR since cells are essentially transparent in the NIR regime.²³ In this work, the nanoantenna surface is modified with a cationic phospholipid bilayer,⁵ and negatively charged siRNA is adsorbed to the surface. Optically generated, surface-localized heat is used to emit siRNA from the nanoantenna. The calculated temperature profiles (Figure S1) show this heat generated is highly localized to the surface and decays exponentially within 100 nm. The calculated concentration profiles in Figure 1b, ii, show siRNA is emitted from the nanoantenna when the antenna effect is “on” at the resonance state. We utilized this resulting outcome of the antenna effect to design siRNA-Au nanoantennas that can be optically addressed to emit siRNA with minimal antenna crosstalk for use in constructing photonic gene circuits.

The siRNA-Au nanoantennas were synthesized and experimentally characterized as functional optical receivers and biomolecular emitters of siRNA (further information in Methods and Figures S2–S8). Inside living cells, interfering siRNA silences intracellular genes in a sequence-specific manner, but alone, lacks the temporal control necessary for precise modulation. The siRNA-Au nanoantennas combine the benefits of sequence-specificity with spatiotemporal control.

Dark-field microscopy was used to visualize internalized siRNA-Au nanoantennas (Figure 2a). Human cervical carcinoma HeLa cells were illuminated with unpolarized white light from an oblique angle using a dark-field condenser lens, and scattered light was collected using a transmission-mode dark-field microscope. To locate cells' boundaries and nuclei, DIC images were overlaid with DAPI-stained images and placed adjacent to dark-field scattering images. Using dark-field microscopy, scattered light from individual nanoantennas can be visualized.^{27,28} In Figure 2c, individual nanoantennas appear uniformly distributed in the cytosol of HeLa cells. It is clearly evident from Figure 2 that scattered light from cells containing nanoantennas (Figure 2c) is easily differentiated from

cells lacking nanoantennas (Figure 2b). Long-term viability/cytotoxicity and proliferation studies were previously conducted to ensure that internalized nanoantennas⁵ and optical excitation⁴ caused no adverse effects on cell behavior.

The circuit diagram for an *OFF*-switch photonic gene circuit is shown in Figure 2d, i. Gene *X* (*gX*) is transcribed into mRNA *X* (*mX*), which is then translated into protein *X* (*pX*). The siRNA-Au nanoantenna is introduced into the circuit diagram as an optical input. Circuit connections are notated in the legend in Figure 1a. When the siRNA-Au nanoantenna is optically addressed, siRNA targeting *X* is emitted from the siRNA-Au nanoantenna, thereby turning off *mX* and subsequently *pX* due to optical gene silencing of *X* (Figure S9). To experimentally demonstrate the *OFF*-switch circuit in HeLa cells, the activated isoform nuclear factor κ B-p65 (p65) was chosen to represent *X*, and flow cytometric analysis was used to quantify the *OFF*-switch of p65 (*i.e.*, decrease in p65 protein levels) in single HeLa cells. In Figure 2d, p65 protein levels decreased by $\Delta = 40\%$ as a result of the *OFF*-switch photonic gene circuit. No decrease in p65 protein levels was observed when optically addressed, nonresonant siRNA-Au nanoantennas failed to emit p65 siRNA (Figure 2d, ii). No decrease in p65 protein levels were observed when optically addressed siRNA-Au nanoantennas emitted scrambled siRNA (Figure 2d, iii). It was observed in the positive control (Figure S10a) that siRNA alone targeting p65 decreased protein expression levels by 60%. We surmise that the difference between optical gene silencing and the positive control could be due to the efficiency of siRNA escaping out of endosomes and into the cytosol after photothermal destabilization of the endosomal membrane. Future in-depth imaging studies of photothermal destabilization of endosomal membranes should help to better understand and improve efficiencies of siRNA escape from photothermally destabilized endosomes. A logic table summarizing the *OFF*-switch behavior is shown in Figure 2e, where “0” (off-state) and “1” (on-state) represent low and high concentrations, respectively. In Figure 2e, HeLa cells immunostained for p65 showed an overall high fluorescence when the input was “0” and an overall low fluorescence when the input was “1” (optically addressed *OFF*-switch). *OFF*-switch photonic gene circuits presented in Figure 2 are not limited to p65 and can be constructed for virtually any protein of interest. *OFF*-switch photonic gene circuits are also modular and can be combined with other naturally occurring subcircuits and/or other photonic subcircuits.

We take advantage of this modularity to construct an *ON*-switch photonic gene circuit. In the circuit diagram in Figure 3b, genes (*gX*, *gY*) are transcribed into mRNA (*mX*, *mY*) and are then translated into proteins (*pX*, *pY*), where *pY* inhibits the active form of *pX*, represented by pX_{nuc} , by sequestering pX_{nuc} in the

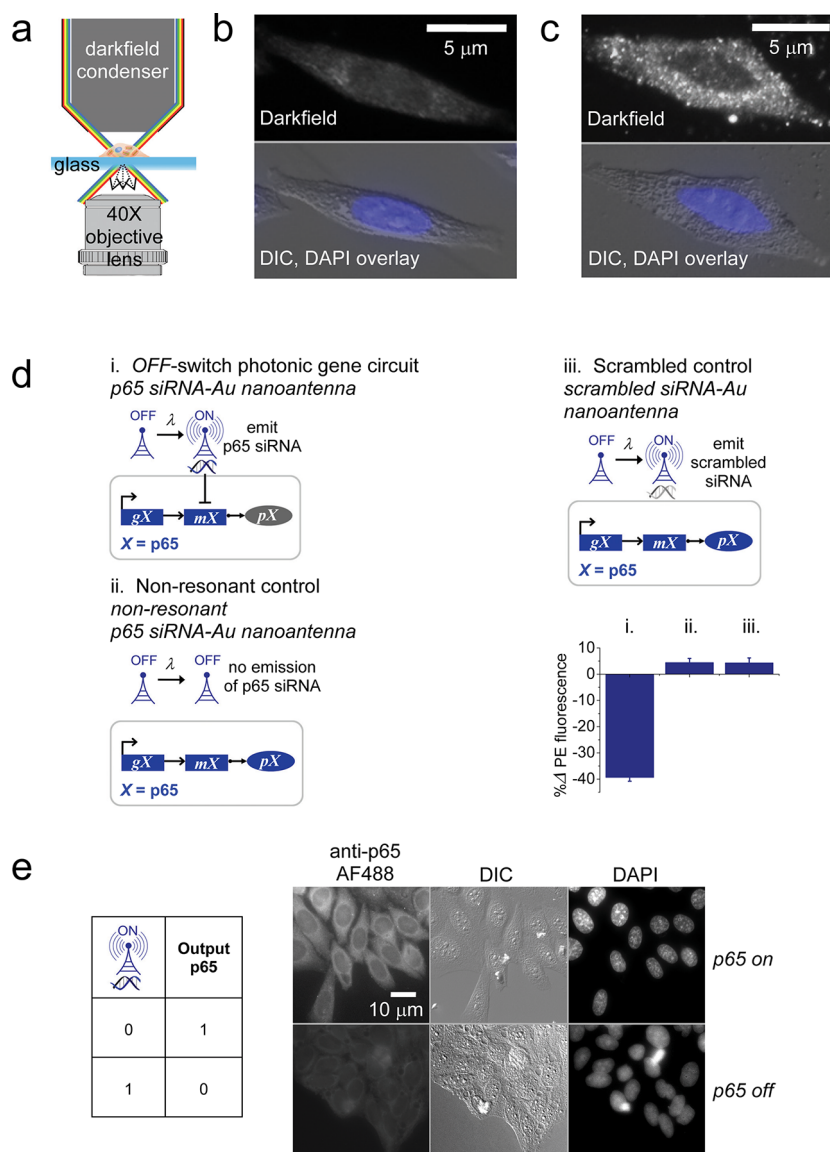


Figure 2. OFF-switch photonic gene circuit. (a) Schematic of microscope setup to visualize internalized siRNA-Au nanoantennas in HeLa cells. (b) Dark-field scattering images of HeLa cells lacking nanoantennas (control). DAPI-stained nuclei images overlaid with DIC images. (c) Dark-field scattering images of HeLa cells with internalized nanoantennas. DAPI-stained nuclei images overlaid with DIC images. (d) Flow cytometric analysis of p65 OFF-switch photonic gene circuit in single HeLa cells. HeLa cells immunostained using phycoerythrin (PE)-labeled anti-p65. Flow cytometric data expressed as percent change of mean PE fluorescence intensity between experiment and reference cells (further information in Figure S10) for OFF-switch photonic gene circuit, nonresonant control, and scrambled control. (i) Circuit diagram for p65 OFF-switch photonic gene circuit. (ii) Circuit diagram for nonresonant control. (iii) Circuit diagram for scrambled control. (e) Logic table for OFF-switch photonic gene circuit. Immunofluorescence imaging of p65 OFF-switch photonic gene circuit in HeLa cells: DIC, anti-p65-AF488 immunostaining of p65, and DAPI staining of nuclei.

cytoplasm (off-state). To construct an ON-switch of pX_{nuc} , a modular OFF-switch subcircuit is introduced into this circuit diagram to generate a double-negative signal (i.e., inhibit the inhibitor pY). Therefore, when the siRNA-Au nanoantenna is optically addressed, pY is turned off, thereby enabling pX_{nuc} to turn on and freely translocate to the nucleus (on-state). To experimentally demonstrate this ON-switch circuit configuration in HeLa cells, p65 was chosen to represent X and the inhibitor κB ($\text{l}\kappa\text{B}$) was selected to represent Y . First, flow cytometric analysis was used to quantify the $\text{l}\kappa\text{B}$ OFF-switch subcircuit (i.e., decrease in $\text{l}\kappa\text{B}$ protein levels) in

single HeLa cells. In Figure 3a, $\text{l}\kappa\text{B}$ protein levels decreased by $\Delta = 40\%$ as a result of the OFF-switch subcircuit. Having confirmed the $\text{l}\kappa\text{B}$ OFF-switch subcircuit, the ON-switch of $p65_{nuc}$ was studied using immunofluorescence imaging. A logic table summarizing the ON-switch behavior is shown in Figure 3b. In the off-state, $\text{l}\kappa\text{B}$ and p65 form an inactive complex such that p65 is sequestered in the cytoplasm. Therefore, HeLa cells immunostained for p65 showed an overall low fluorescence in the nucleus when the input was "0". Conversely, cells showed a strong nuclear presence of p65 ($p65_{nuc}$) when the input was "1"

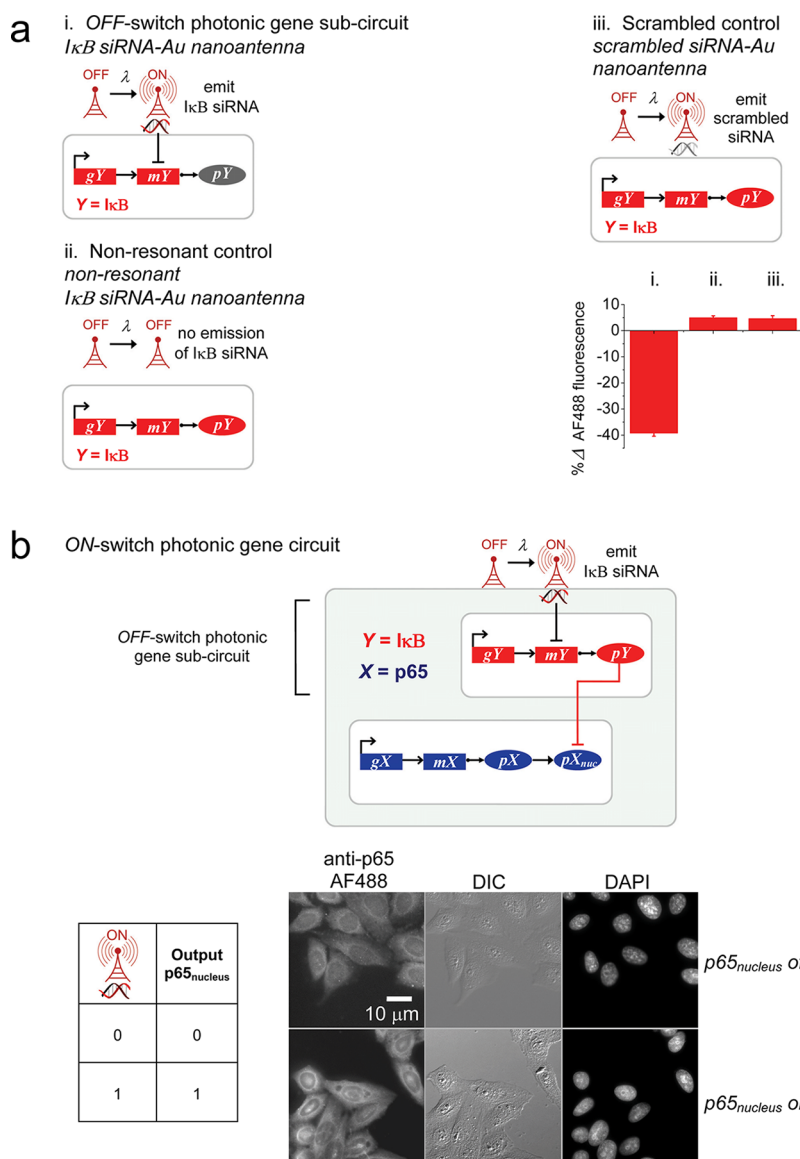


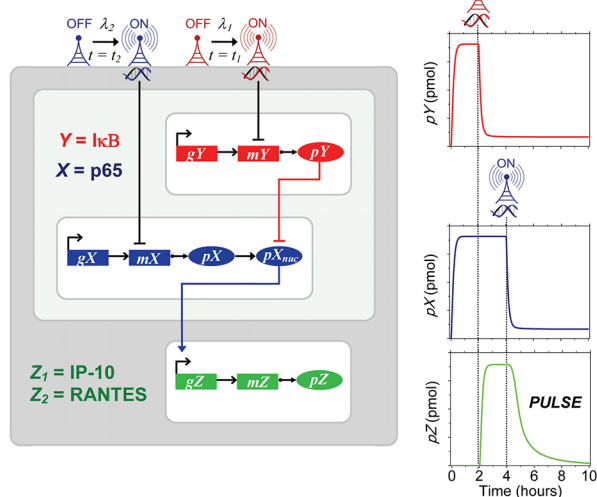
Figure 3. ON-switch photonic gene circuit. ON-switch photonic gene circuit is constructed using a modular OFF-switch subcircuit. IkB and p65 were chosen to represent Y and X, respectively. (a) Flow cytometric analysis of IkB OFF-switch subcircuit in single HeLa cells. HeLa cells immunostained using AF488-labeled anti-IkB. Flow cytometric data expressed as percent change of mean AF488 fluorescence intensity between experiment and reference cells (further information in Figure S11) for IkB OFF-switch subcircuit, nonresonant control, and scrambled control. (i) Circuit diagram for IkB OFF-switch subcircuit. (ii) Circuit diagram for nonresonant control. (iii) Circuit diagram for scrambled control. (b) Logic table for ON-switch photonic gene circuit. In the on-state, p65 translocates to the nucleus ($p65_{nucleus}$). Immunofluorescence imaging of ON-switch photonic gene circuit in HeLa cells: DIC, anti-p65-AF488 immunostaining of p65, and DAPI staining of nuclei.

(optically addressed ON-switch). To locate cells' boundaries and nuclei, DIC images and DAPI images were placed adjacent to p65 immunostained images. Similar to OFF-switch circuits, ON-switch photonic gene circuits are also modular and can potentially be combined with multiple subcircuits to construct more sophisticated photonic gene circuits.

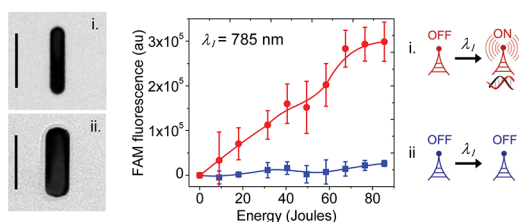
To construct more sophisticated photonic gene circuits from multiple subcircuits, modular subcircuits should function independently of each other. This can be accomplished with siRNA-Au nanoantennas that operate at distinct optical wavelengths (Figure 4b), based on aspect ratio (AR), with minimal antenna crosstalk. Selective optical release of two distinct

DNA strands has been previously demonstrated,¹⁵ but not yet in the context of engineering gene circuits. Fluorescence analysis was conducted to measure antenna crosstalk between 4.0 AR and 2.5 AR siRNA-Au nanoantennas. Using $\lambda_1 = 785$ nm, a statistically significant increase in fluorescence intensity was seen when optically addressed resonant siRNA-Au nanoantennas (4.0 AR) emitted fluorescently labeled siRNA, while no increase in fluorescence intensity was observed when nonresonant nanoantennas (2.5 AR) were optically addressed (Figure 4b, Figure S6a), indicating no antenna crosstalk using $\lambda_1 = 785$ nm. It was observed that when using $\lambda_2 = 660$ nm (Figure S6b), a statistically significant increase in the fluorescent

a PULSE-switch photonic gene circuit



b



c

	ON	ON	Output IP-10 early gene	Output RANTES late gene
i.	0	0	0	0
ii.	0	1	0	0
iii.	1	0	1	1
iv.	1 ($t_1 = 0$ hr)	1 ($t_2 = 2$ hr)	1	0

d

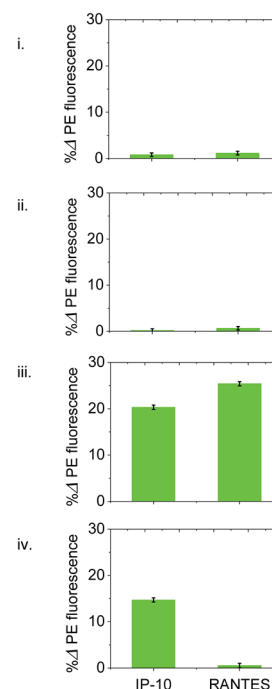


Figure 4. PULSE-switch photonic gene circuit. (a) A PULSE-switch photonic gene circuit is constructed using multiple, independently operating subcircuits. Circuit diagram and model for PULSE-switch photonic gene circuit. The ON-switch subcircuit functions to initiate the pulse of target protein pZ at time t_1 . In this ON-switch subcircuit, siRNA-Au nanoantennas (4.0 AR) are optically addressed at time t_1 using $\lambda_1 = 785$ nm. The OFF-switch subcircuit then operates to terminate the pulse of target pZ at time t_2 . In this OFF-switch subcircuit, siRNA-Au nanoantennas (2.5 AR) are optically addressed at time t_2 using $\lambda_2 = 660$ nm. IkB and p65 were chosen to represent Y and X, respectively. Two targets, IP-10 and RANTES, were selected to represent Z (as Z_1 and Z_2 , respectively). (b) Photonic gene subcircuits operate independently of each other using optically addressable siRNA-Au nanoantennas with minimal antenna crosstalk. Fluorescence analysis of fluorescently labeled siRNA emitted from optically addressed resonant siRNA-Au nanoantennas (further information in Figure S6). (i) Transmission electron microscopy image of the 4.0 AR nanoantenna structure (gold material). (ii) Transmission electron microscopy image of the 2.5 AR nanoantenna structure (gold material). Scale bar is 50 nm. (c) Logic table for 2 h PULSE-switch photonic gene circuit in single HeLa cells corresponding to logic table. HeLa cells immunostained using phycoerythrin (PE)-labeled anti-IP-10 or PE-labeled anti-RANTES. Flow cytometric data expressed as percent change of the mean PE fluorescence intensity between experiment (conditions listed in logic table) and reference cells (further information in Figure S12).

intensity was seen with resonant nanoantennas (2.5 AR), and a small but measurable increase in fluorescent intensity was also observed with nonresonant nanoantennas (4.0 AR). In this case, signal interference occurred with nonresonant nanoantennas, but this can be circumvented in the future by designing nanoantennas to achieve a wider spectral separation between their longitudinal plasmon resonance bands.

Having confirmed that siRNA-Au nanoantennas can selectively receive optical signals and preferentially emit siRNA with minimal antenna crosstalk, we constructed a PULSE-switch photonic gene circuit from multiple, independently operating subcircuits. In the circuit diagram in Figure 4a, ON-switch and OFF-switch subcircuits are combined to form a PULSE-switch circuit. The ON-switch subcircuit functions to initiate the pulse of target protein pZ at time t_1 . In this ON-switch

subcircuit, siRNA-Au nanoantennas (4.0 AR) are optically addressed at time t_1 using $\lambda_1 = 785$ nm to emit siRNA targeting Y. This causes pY to turn off, pX_{nuc} to turn on, and subsequently target pZ to turn on. The OFF-switch subcircuit then operates to terminate the pulse of target pZ at time t_2 . In this OFF-switch subcircuit, siRNA-Au nanoantennas (2.5 AR) are optically addressed at time t_2 using $\lambda_2 = 660$ nm to emit siRNA targeting X. This causes pX to turn off and subsequently target pZ to turn off. ON-switch and OFF-switch subcircuits should operate separately, with minimal signal interference from the other circuit. However, since some signal interference was observed with $\lambda_2 = 660$ nm (Figure S6b) while no signal interference was observed with $\lambda_1 = 785$ nm, here, the order of operation became critical. The ON-switch was first designed to operate at $\lambda_1 = 785$ nm since no signal interference

occurred using $\lambda_1 = 785$ nm (Figures 4b and S6a). The OFF-switch was then designed to operate at $\lambda_2 = 660$ nm. In this order of operation, both circuits were able to operate independently of each other. In future versions, signal interference will be minimized by designing nanoantennas with a wider spectral separation between their longitudinal plasmon resonance bands or by designing new geometries of nanoantennas with narrower spectral bandwidths.

To experimentally demonstrate this *PULSE*-switch photonic gene circuit in HeLa cells, I κ B and p65 were chosen to represent *Y* and *X*, respectively. To test the kinetic behavior of the *PULSE*-switch photonic gene circuit, temporally regulated genes were used as a functional assay of kinetics. Two NF- κ B regulated genes (IP-10 and RANTES) that display different transcriptional activation profiles were selected to represent *Z* (as *Z*₁ and *Z*₂, respectively). IP-10, an early response gene, is known to be immediately activated within 30 min of stimulation,²⁹ whereas RANTES, a late response gene, requires more than 3 h of stimulation to turn on.³⁰ We used these two temporally regulated genes to functionally validate the 2 h *PULSE*-switch shown in Figure 4a. We reasoned that if a 2 h *PULSE*-switch was constructed, IP-10 should turn on while RANTES should remain off. To implement the 2 h *PULSE*-switch, we constructed a logic table for IP-10 and RANTES summarizing four possible conditions (Figure 4c). These conditions listed in the logic table were shown to function correctly using flow cytometric analysis of IP-10 and RANTES (Figure 4d). Notably, Figure 4d, iv, confirmed that IP-10 is turned on while RANTES is turned off as a result of the 2 h *PULSE*-switch. In addition to functional assays of kinetics, visual assays using fluorescently labeled proteins can also be utilized in the future to characterize photonic gene circuits.

In this work, we have demonstrated the construction of a *PULSE*-switch circuit using two independent subcircuits. This was accomplished with two independently operating nanoantennas. In the future, more sophisticated circuit configurations can be conceivably constructed by combining multiple, independent subcircuits. Due to the narrow spectral bandwidths of Au nanorods, four subcircuits could potentially operate independently of each other in the visible and

near-infrared wavelength regimes. More subcircuits could potentially be combined by synthesizing new geometries of nanoantennas with more narrow spectral bandwidths in the visible and near-infrared wavelength regimes. As more subcircuits are combined, signal strength should be addressed to improve circuit performance. Signal distortions, such as amplitude reductions, occur due to inherent dilution, degradation, and diffusion effects in the intracellular environment. These signal distortions inherently occur in native gene circuits. Inevitably, photonic gene circuits are subject to similar signal distortions as native gene circuits. Improving RNA interference efficiency could help to overcome the effects of signal amplitude reduction. For example, designing siRNA to bind to optimal target sites could help to improve RNA interference efficiency. Incorporating other geometries of RNA, such as hairpin-shaped or dumbbell-shaped RNA, with nanoantennas, which may be less prone to degradation, could also help to improve RNA interference efficiency in the intracellular environment.

CONCLUSIONS

In closing, we have demonstrated the construction of photonic gene circuits using optically addressable siRNA-Au nanoantennas in living cells. Photonic gene circuits are a promising approach to systematically study native gene circuits in complex, naturally occurring living systems. Since native gene circuits remain genomically unaltered, photonic gene circuits are a promising alternative to synthetic circuits for studying temporal dynamics³¹ in native gene circuits. Toward *in vivo* applications, we envision constructing photonic gene circuits using optically addressable siRNA-Au nanoantennas in optically transparent whole model organisms, such as *Caenorhabditis elegans* and zebrafish embryos, to study temporal dynamics and to probe, identify, and reconfigure malfunctioning gene circuits as a therapeutic strategy. We envision that light also could be delivered transdermally or by fiber optics in other model organisms that are not optically transparent. We believe photonic gene circuits could play a pivotal role in engineering functional gene circuits as they enable, for the first time, dynamic reconfiguration and temporally precise modulation of native gene circuits.

METHODS

Modeling of Optical Receiver. The finite element method (COMSOL Multiphysics software) was used to model a 3-D Au nanorod (4.0 and 2.5 AR) suspended in water and to achieve a solution to the Helmholtz wave equation: $\nabla \times (\mu_r^{-1} \nabla \times E) - k_0^2 (\epsilon_r - j\sigma\omega\epsilon_0)E = 0$. The Au nanorod was constructed using a cylinder with hemispheres on each end of the cylinder. The relative permeability of Au was assumed to be $\mu_r = 1$, and the complex permittivity of Au ϵ_r was assumed to be a function of wavelength λ . A spherical perfectly matched layer and an integration layer, modeled by concentric spheres, were used

to reach perfect absorption at the outer boundary and minimize spurious reflections. A plane wave was used for excitation ($\lambda = 780$ nm or $\lambda = 650$ nm). The adaptive mesh was refined until the maximum electric field converged.

Modeling of Optically Generated Surface Localized Heat. We solved the bioheat transfer equation: $\rho C(\partial T/\partial t) + \nabla \cdot (-k \nabla T) = \rho_b C_b \omega_b (T_b - T) + Q_{\text{met}} + Q_{\text{ext}}$. Time-average resistive heat (W/m^3) was used for the spatial heat source Q_{ext} under the assumption that electromagnetic energy was converted to heat by resistive heating. The thermal conductivity of gold, density of gold, and specific heat of gold were assumed to be $k_g = 320$ W/m-K,

$\rho_g = 19\,300\text{ kg/m}^3$, and $C_g = 129\text{ J/kg-K}$, respectively.^{32,33} The thermal conductivity of water, density of water, and specific heat of water were assumed to be $k_w = 0.61\text{ W/m-K}$, $\rho_w = 1000\text{ kg/m}^3$, and $C_w = 4200\text{ J/kg-K}$, respectively. The metabolic heat source Q_{met} and the perfusion rate ω_b were assumed to be insignificant.

Modeling of Biomolecular Emitter. To investigate biomolecular emission of siRNA, we solved the thermal diffusion equation: $\partial c/\partial t = \nabla \cdot [D \nabla c + D_T c \nabla T]$. Electromagnetic energy was assumed to be converted to surface-localized heat and was used here for temperature T . It was assumed that siRNA dissociated from the surface of the nanoantenna at elevated temperatures, and mass flux occurred due to the temperature gradient in addition to the siRNA concentration gradient. The diffusion coefficient and the thermal diffusion coefficient of siRNA were estimated to be $D = 36.00 \times 10^8\text{ cm}^2/\text{s}$ and $D_T = 0.45 \times 10^8\text{ cm}^2/\text{s-K}$, respectively, based on double-stranded DNA of similar length.³⁴ Using the surface density ($9.0 \times 10^{12}\text{ molecules/cm}^2$) and the size dimensions, the initial concentrations of siRNA were calculated to be $c_0 = 1.81 \times 10^{-14}\text{ mol/m}^3$ for 4.0 AR nanoantennas and $c_0 = 2.11 \times 10^{-14}\text{ mol/m}^3$ for 2.5 AR nanoantennas, respectively.

Modeling of Photonic OFF-Switch, ON-Switch, and PULSE-Switch Gene Circuits. The OFF-switch (eqs 1–3), ON-switch (eqs 4–8), and PULSE-switch (eqs 9–17) circuit configurations were modeled (Matlab software). Genes (gX , gY , gZ) were transcribed into mRNA (mX , mY , mZ), which were then translated into proteins (pX , pY , pZ), where gX , gY , gZ , mX , mY , mZ , pX , pY , and pZ denote concentrations. Degradation of messenger mRNA by optical gene silencing using gold nanoantennas functionalized with siRNA (NA_{siRNA1} , NA_{siRNA2}) were modeled based on Michaelis–Menten/Hill kinetics.

OFF-Switch Circuit

$$\frac{dmX}{dt} = k_{X1} \cdot gX - k_{d-mX} \cdot mX - k_{d-sirna2-mrna} \cdot \beta_X \cdot \left(\frac{NA_{\text{on}} \text{siRNA2}}{K_X + NA_{\text{on}} \text{siRNA2}} \right) \cdot mX \quad (1)$$

$$\frac{dpX}{dt} = k_{X2} \cdot mX - k_{d-pX} \cdot pX \quad (2)$$

$$\frac{dNA_{\text{on}} \text{siRNA2}}{dt} = k_{L2} \cdot NA_{\text{siRNA2}} - k_{d-sirna2} \cdot NA_{\text{on}} \text{siRNA2} \quad (3)$$

ON-Switch Circuit

$$\frac{dmY}{dt} = k_{Y1} \cdot gY - k_{d-mY} \cdot mY - k_{d-sirna1-mrna} \cdot \beta_Y \cdot \left(\frac{NA_{\text{on}} \text{siRNA1}}{K_Y + NA_{\text{on}} \text{siRNA1}} \right) \cdot mY \quad (4)$$

$$\frac{dmX}{dt} = k_{X1} \cdot gX - k_{d-mX} \cdot mX \quad (5)$$

$$\frac{dpX}{dt} = k_{X2} \cdot mX - k_{d-pX} \cdot pX \quad (6)$$

$$\frac{dpX_{\text{nuc}}}{dt} = k_{X3} \cdot pX - k_{d-pX_{\text{nuc}}} \cdot pX_{\text{nuc}} - k_{d-pX_{\text{nuc}}-pY} \cdot pY \cdot pX_{\text{nuc}} \quad (7)$$

$$\frac{dNA_{\text{on}} \text{siRNA1}}{dt} = k_{L1} \cdot NA_{\text{siRNA1}} - k_{d-sirna1} \cdot NA_{\text{on}} \text{siRNA1} \quad (8)$$

PULSE-Switch Circuit

$$\frac{dmY}{dt} = k_{Y1} \cdot gY - k_{d-mY} \cdot mY - k_{d-sirna1-mrna} \cdot \beta_Y \cdot \left(\frac{NA_{\text{on}} \text{siRNA1}}{K_Y + NA_{\text{on}} \text{siRNA1}} \right) \cdot mY \quad (9)$$

$$\frac{dpY}{dt} = k_{Y2} \cdot mY - k_{d-pY} \cdot pY \quad (10)$$

$$\frac{dmX}{dt} = k_{X1} \cdot gX - k_{d-mX} \cdot mX - k_{d-sirna2-mrna} \cdot \beta_X \cdot \left(\frac{NA_{\text{on}} \text{siRNA2}}{K_X + NA_{\text{on}} \text{siRNA2}} \right) \cdot mX \quad (11)$$

$$\frac{dpX}{dt} = k_{X2} \cdot mX - k_{d-pX} \cdot pX \quad (12)$$

$$\frac{dpX_{\text{nuc}}}{dt} = k_{X3} \cdot pX - k_{d-pX_{\text{nuc}}} \cdot pX_{\text{nuc}} - k_{d-pX_{\text{nuc}}-pY} \cdot pY \cdot pX_{\text{nuc}} \quad (13)$$

$$\frac{dmZ}{dt} = k_{Z1} \cdot gZ \cdot \beta_Z \cdot \left(\frac{pX_{\text{nuc}}^n}{K_Z + pX_{\text{nuc}}^n} \right) - k_{d-mZ} \cdot mZ \quad (14)$$

$$\frac{dpZ}{dt} = k_{Z2} \cdot mZ - k_{d-pZ} \cdot pZ \quad (15)$$

$$\frac{dNA_{\text{on}} \text{siRNA1}}{dt} = k_{L1} \cdot NA_{\text{siRNA1}} - k_{d-sirna1} \cdot NA_{\text{on}} \text{siRNA1} \quad (16)$$

$$\frac{dNA_{\text{on}} \text{siRNA2}}{dt} = k_{L2} \cdot NA_{\text{siRNA2}} - k_{d-sirna2} \cdot NA_{\text{on}} \text{siRNA2} \quad (17)$$

Kinetic rates and initial values are listed in Supporting Information Table 1 and were approximated similarly to those previously reported in the literature for a eukaryotic model.³⁵ It was assumed that no siRNA was emitted by nonresonant siRNA-Au nanoantennas. It was also assumed that when siRNA-Au nanoantennas were optically addressed with the correct optical wavelength, all siRNA was emitted. Since the model's time scale was on the order of hours, it was assumed that nanoantennas did not degrade in this time frame, and therefore the concentration of nanoantennas remained the same. Due to the tight packing of siRNA on the nanoantennas, steric hindrances inhibited nuclease degradation³⁶ of siRNA while siRNA was bound to the nanoantennas. Once siRNA was emitted from nanoantennas, the siRNA degraded with degradation rates as reported previously.³⁷

Synthesis of RNase-Free Gold Rod-Shaped Nanoantennas. Gold nanorods of aspect ratios 2.5 and 4.0 (Figure S2) were synthesized by adapting a seed-mediated growth method^{38,39} to an RNase-free environment.⁵ All solutions were prepared using 0.2 μm filtered DEPC-treated water. All glassware and metalware were baked at 240 $^{\circ}\text{C}$ for 24 h to remove exogenous RNases. All pipetting devices and counter space was treated with 70% ethanol. All disposable plastic pipet tips and centrifuge tubes were certified to be free of RNase. The resultant CTAB-coated gold nanoantennas were ensured to be free of RNases by detecting RNase activity over time (Figure S3). RNase activity in the supernatant solution was detected using an RNase activity kit (Ambion) and was quantitatively measured over time using a fluorometer (Fluoromax-3, Horiba Jobin Yvon). The concentration of CTAB-coated nanorods was confirmed by adjusting to an absorbance of 1 at the longitudinal plasmon resonance wavelength using UV–vis spectroscopy (8453, Hewlett-Packard). Aspect ratios were determined by scanning electron microscopy and transmission electron microscopy.

Synthesis of siRNA-Au Nanoantennas. Gold nanoantennas functionalized with siRNA were synthesized as previously described.⁵ In summary, the CTAB surfactant on the nanoantennas' surface was exchanged with a cationic phospholipid bilayer to form biologically functional cationic phospholipid–gold nanoantennas. To remove excess CTAB surfactant, 500 μL of unmodified CTAB-coated nanoantennas (UV–vis absorbance of 1) was centrifuged at 5000 rpm for 10 min. A 10 μL pellet was transferred to a new microcentrifuge tube, redispersed in 500 μL of nuclease-free water, briefly vortexed, and sonicated for 1 min. To replace CTAB surfactant with a phospholipid bilayer membrane at the nanoantenna surface, nanoantennas were then centrifuged again at 5000 rpm for 10 min. A 10 μL pellet was

transferred to a new microcentrifuge tube, resuspended in 50 μ L of Oligofectamine, briefly vortexed, and sonicated for 1 min.

After the CTAB coating was replaced with a cationic phospholipid coating, siRNA was then conjugated to the nanoantennas. kB siRNA (Qiagen) was conjugated to nanoantennas (4.0 AR), and p65 siRNA (Qiagen) was conjugated to nanoantennas (2.5 AR). To 500 μ L of nanoantenna solution, 2 μ L of 100 μ M siRNA was added. The solution was vortexed and allowed to incubate for 30 min. To remove excess siRNA from solution, nanoantennas were washed with nuclease-free water by centrifugation at 5000 rpm for 10 min and finally resuspended in 500 μ L of nuclease-free water. After preparation of siRNA-Au nanoantennas, an absorbance of 0.2 was measured by UV-vis (8453, Hewlett-Packard). By comparing with the original nanoantennas' UV-vis absorbance of 1, the concentration of siRNA-Au nanoantennas was estimated to be approximately one-fifth the original concentration (approximately 6 μ g/mL or 1.4×10^{11} nanoantennas/mL).

For internalization, the siRNA-Au nanoantennas were then coated with cationic phospholipids to improve entry into living cells. First, 1000 μ L of siRNA-Au nanoantennas was concentrated into a 10 μ L pellet by centrifugation at 5000 rpm for 20 min. The pellet was transferred to a new microcentrifuge tube and then resuspended in 25 μ L of Oligofectamine. The solution was vortexed and allowed to incubate for 20 min. For higher concentrations of conjugates, the multiple tubes of conjugates were prepared the same as described above. After 20 min, 175 μ L of nuclease-free water was added to each tube to dilute the concentration of free cationic phospholipids in solution. The conjugates were concentrated into a 0.5 μ L pellet by centrifugation for internalization.

Thermal Liberation of siRNA from siRNA-Au Nanoantennas. Prior to optically addressing siRNA-Au nanoantennas, the controlled thermal liberation of siRNA from the surface was first demonstrated. If siRNA dissociates from the cationic phospholipid bilayer at elevated temperatures, this unbinding event should change the dielectric constant of the medium locally surrounding the nanoantennas and therefore result in an observable shift in the nanoantennas' longitudinal plasmon resonance wavelength.^{40,41}

A UV-vis spectrometer containing a thermal-jacketed cell was used to evaluate the temperature-dependent absorbance of the nanoantenna-containing solutions. To each separate cell of an eight-cell micro sample holder (208-92086, Shimadzu) was added 50 μ L of siRNA-Au nanoantennas or unconjugated nanoantennas. Samples were simultaneously heated from 20 to 70 $^{\circ}$ C (10 $^{\circ}$ C increment, 5 min) using a temperature-controlled UV-vis spectrometer (2501, Shimadzu). Absorbance spectra were collected for each sample at each temperature increment.

Control nanoantennas lacking siRNA cargo showed no shift in the longitudinal plasmon resonance wavelength at elevated temperatures (Figure S4b), indicating that the phospholipid bilayer remained stable at elevated temperatures. In contrast, siRNA-Au nanoantenna conjugates showed a marked blue-shift in the longitudinal plasmon resonance wavelength at elevated temperatures due to the dissociation of siRNA from the surface (Figure S4a). Additionally, supplemental Figure S4c shows that the longitudinal plasmon resonance wavelength of the siRNA-Au nanoantennas blue-shifts with incrementing temperature until it eventually matches the longitudinal plasmon resonance wavelength of the control nanoantennas, strongly suggesting the complete dissociation of siRNA from the intact phospholipid bilayer at 70 $^{\circ}$ C.

Optical Setup. An 80 mW 785 nm CW diode laser (Newport Corp.) and a 60 mW 660 nm CW diode laser (Newport Corp.) were optically coaligned such that their beams simultaneously overlapped (Figure S5). First, both lasers were positioned parallel to each other on a manually adjustable xyz stage. A dichroic mirror (Omega Optical) was positioned below the 785 nm laser. A mirror (Omega Optical) was placed below the 660 nm laser. To make the two beams orthogonal to each other, the 660 nm laser beam was reflected 90 $^{\circ}$ toward the direction of the other laser by the mirror. The dichroic mirror transmitted the 785 nm laser beam and reflected the 660 nm laser beam another 90 $^{\circ}$ such that both beams simultaneously overlapped and coaligned

with each other. To circularly polarize the light, an achromatic quarter-wave plate (CVI Laser Corp.) was placed below the coaligned beams.

Emission of siRNA from Optically Addressed siRNA-Au Nanoantennas. Having established that siRNA can be thermally dissociated from the cationic phospholipid bilayer, siRNA-Au nanoantennas of different aspect ratios were then selectively addressed. Because their narrow longitudinal plasmon resonance bands are spectrally separated (Figure S2a), rod-shaped Au nanoantennas can be optically addressed to selectively emit siRNA.

6-Carboxyfluorescein (FAM; excitation 495 nm, emission 520 nm)-labeled siRNA was bound to the nanoantennas, and unbound FAM-siRNA was then removed from the background solution by centrifugation. To a 2 mm path length quartz cuvette (3-2.45-Q-2, Starna Cells Inc.) were added 50 μ L of FAM-siRNA-Au nanoantennas (2.5 AR) or FAM-siRNA-Au nanoantennas (4.0 AR). Using the optical setup shown in Figure S5, samples were illuminated from the top with 50 mW of either 785 or 660 nm light with a spot size of 2 mm. During illumination, fluorescence emission spectra were collected at 2 min intervals for each sample using a fluorometer (Fluoromax-3, Horiba Jobin Yvon). A short-pass filter was positioned in front of the fluorometer's detector to block the detector from the laser sources.

Concentration Calibration of Optically Liberated siRNA. To calibrate the fluorescent intensity to the concentration of siRNA liberated from nanoantenna carriers into solution, the fluorescent intensities of known concentrations of FAM-siRNA were also measured (Figure S7). As seen in supporting Figure S7, nanoantennas (concentration 1×10^{11} nanoantennas/mL based on UV-vis measurements) released approximately 0.05 μ M siRNA after illumination. As a positive control, Triton X-100 detergent was used to disrupt the cationic phospholipid bilayer around the nanoantennas, thereby ensuring the complete liberation of all bound siRNA from nanoantennas into solution. The concentration of siRNA released using Triton X-100 matched closely the concentration of optically liberated siRNA, strongly suggesting complete liberation of siRNA from optically addressed nanoantennas.

Cell Preparation. The human cervical carcinoma cell line HeLa was purchased from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's media (DMEM) formulated with high glucose and GlutaMAX was purchased from Invitrogen and was supplemented with 10% heat-inactivated fetal bovine serum. Cells were seeded at an initial concentration of 20 000 cells/well in a 96-well plate, cultured in the supplemented media, and maintained in a 37 $^{\circ}$ C incubator with 5% CO₂ humidified air.

Visualization of Internalized siRNA-Au Nanoantennas in HeLa Cells. siRNA-Au nanoantennas were internalized into HeLa human cervical cancer cells. For visualization purposes, HeLa cells were seeded onto 12 mm gridded glass coverslips (Electron Microscopy Sciences) at 30 000 cells/well in a 24-well plate for 24 h before use. HeLa cells were then washed once with Optimum media. The 0.5 μ L concentrated pellet of siRNA-Au nanoantennas was resuspended in 100 μ L of Optimum media, gently mixed, and added to each well of the 24-well plate. The cells were allowed to incubate for 4 h at 37 $^{\circ}$ C. Cells were then fixed by incubating cells with 2% paraformaldehyde (Electron Microscopy Sciences) in 1 \times PBS per well for 10 min. Cell nuclei were stained with DAPI (Invitrogen) by incubating cells in 300 nM DAPI in 1 \times PBS per well for 5 min. 1 \times PBS was used to twice wash the cells. The coverslip containing fixed, adhered cells was then placed face down and adhered to a microscope slide. Cells were located using the grids imprinted on the coverslips.

Dark-field microscopy was used to visualize internalized siRNA-Au nanoantennas (Figure 2a). Dark-field scattering was visualized using an inverted microscope (Axiovert, Zeiss) at 40 \times magnification. Broadband white light was shone onto the adhered cells from an oblique angle using a dark-field condenser lens. The scattered light alone was collected using a microscope objective lens with a numerical aperture (NA) of 0.65, which was smaller than the NA (1.2–1.4) of the illumination condenser lens. To locate cells' boundaries and nuclei, DIC images were overlaid with DAPI-stained images and placed adjacent to dark-field scattering images. DIC and DAPI were

visualized using an upright fluorescence microscope (Axio Imager, Zeiss) at 40 \times magnification.

Concentration Characterization of Internalized siRNA-Au Nanoantennas in HeLa Cells. To estimate the amount of siRNA liberated from nanoantennas into the intracellular space, fluorescently labeled FAM-siRNA was bound to nanoantennas and unbound FAM-siRNA was then removed from the background solution by centrifugation. Known concentrations of FAM-siRNA-Au nanoantennas (7×10^{11} , 4×10^{11} , and 3×10^{11} nanoantennas/mL based on UV-vis measurements) were internalized in HeLa cells. Fluorescent intensities of individual cells were then measured by flow cytometry, and a standard concentration curve of internalized FAM-siRNA-Au nanoantennas was constructed (Figure S8b). Fluorescence quenching by nanoantennas was not observed.⁵ To correlate the fluorescent intensities to FAM-siRNA concentration, control cells were incubated with known concentrations of FAM-siRNA (50, 100, and 200 nM) for 5 h. A standard concentration curve of internalized FAM-siRNA was then constructed based on flow cytometry analysis (Figure S8a). These standard curves were utilized to estimate the concentration of siRNA-Au nanoantennas necessary for optical gene silencing.

OFF-Switch Photonic Gene Circuit (p65 off). HeLa cells were then washed once with Optimum media. A 0.5 μ L concentrated pellet of siRNA-Au nanoantennas (2.5 AR) functionalized with p65 siRNA was resuspended in 100 μ L of Optimum media, gently mixed, and added to each well of the 96-well plate. The cells were allowed to incubate for 4 h at 37 $^{\circ}$ C. After internalization of siRNA-Au nanoantennas for 4 h, the media was replaced with fresh supplemented DMEM culture media. The 96-well plate was placed in a CO₂-filled, sealed container, containing a high-transmission glass window (Edmund Optics). Wells were illuminated from the top with 50 mW of a 660 nm CW diode laser (Newport Corp.) with a spot size of 2 mm (one quadrant of a well in a 96-well plate) for 15 min. After illumination, cells were allowed to incubate for an additional 72 h at 37 $^{\circ}$ C. Cells were then immunostained for p65 and analyzed by flow cytometry and by immunofluorescence imaging.

ON-Switch Photonic Gene Circuit (p65_{src} on). HeLa cells were then washed once with Optimum media. A 0.5 μ L concentrated pellet of siRNA-Au nanoantennas (4.0 AR) functionalized with κ B siRNA was resuspended in 100 μ L of Optimum media, gently mixed, and added to each well of the 96-well plate. The cells were allowed to incubate for 4 h at 37 $^{\circ}$ C. After internalization of siRNA-Au nanoantennas for 4 h, the media was replaced with fresh supplemented DMEM culture media. The 96-well plate was placed in a CO₂-filled, sealed container, containing a high-transmission glass window (Edmund Optics). Wells were illuminated from the top with 50 mW of a 785 nm CW diode laser (Newport Corp.) with a spot size of 2 mm (one quadrant of a well in a 96-well plate) for 15 min. After illumination, cells were allowed to incubate for an additional 72 h at 37 $^{\circ}$ C. Cells were either immunostained for κ B and analyzed by flow cytometry or immunostained for p65 and analyzed by immunofluorescence imaging.

PULSE-Switch Photonic Gene Circuit (IP-10 on, RANTES off). HeLa cells were then washed once with Optimum media. A 0.5 μ L concentrated pellet of siRNA-Au nanoantennas (4.0 AR) functionalized with κ B siRNA was added to a 0.5 μ L concentrated pellet of siRNA-Au nanoantennas (2.5 AR) functionalized with p65 siRNA and was resuspended in 200 μ L of Optimum media, gently mixed, and added to each well of the 96-well plate. The cells were allowed to incubate for 4 h at 37 $^{\circ}$ C. After internalization of siRNA-Au nanoantennas for 4 h, the media was replaced with fresh supplemented DMEM culture media. The 96-well plate was placed in a CO₂-filled, sealed container, containing a high-transmission glass window (Edmund Optics). Wells were illuminated from the top with 50 mW of a 785 nm CW diode laser (Newport Corp.) with a spot size of 2 mm (one quadrant of a well in a 96-well plate) for 15 min. Two hours after initial illumination with 785 nm light, wells were illuminated from the top with 50 mW of a 660 nm CW diode laser (Newport Corp.) with a spot size of 2 mm (one quadrant of a well in a 96-well plate) for 15 min. After illumination, cells were allowed to incubate for an additional 72 h at 37 $^{\circ}$ C. The media was replaced with fresh supplemented DMEM culture media containing 3 μ M monensin (Sigma) 48 h after illumination. The cells were allowed to incubate

for an additional 24 h at 37 $^{\circ}$ C. Cells were then immunostained for IP-10 or RANTES and analyzed by flow cytometry.

Immunostaining for Flow Cytometry Analysis. Fluorescently labeled antibodies recognizing p65 and κ B were purchased from Santa Cruz Biotechnologies. Fluorescently labeled normal mouse isotype antibodies were also purchased from Santa Cruz Biotechnologies and were used as negative controls. The isotype control and fluorescently labeled antibodies recognizing IP-10 and RANTES were purchased from R&D Systems. Permeabilization buffer, for use with p65 and κ B antibodies, was prepared by adding 0.1% (w/v) saponin, 0.3% (w/v) Triton-X, and 0.1% (w/v) Na₂S₂O₃ to Hank's balanced salt solution (Invitrogen). Permeabilization buffer, for use with IP-10 and RANTES antibodies, was prepared by adding 0.1% (w/v) saponin and 0.06% (w/v) Na₂S₂O₃ to Hank's balanced salt solution (Invitrogen).

Cells were harvested, resuspended in 250 μ L of 1 \times PBS, and fixed with 250 μ L of 4% paraformaldehyde for 10 min. After 10 min, excess paraformaldehyde was removed by centrifuging and resuspending cells in 400 μ L of permeabilization buffer (repeated twice). Cells were then counted to ensure all samples contained the same number of cells prior to immunostaining. For 50 000 cells, 10 μ L of antibody or isotype antibody was added. Cells were gently mixed and incubated at room temperature for 45 min. After 45 min, excess antibodies were removed by centrifuging and resuspending cells in 400 μ L of permeabilization buffer. To remove permeabilization buffer, cells were finally centrifuged and resuspended in 500 μ L of 1 \times PBS. A LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA) were used to analyze samples.

Immunostaining of p65 for Immunofluorescence Imaging. Seventy-two hours after optical gene silencing of κ B or p65, cells (adhered to glass coverslips) were fixed in cold 50% methanol for 3 min on ice followed by cold 100% methanol for 15 min on ice. Cells were washed three times with and incubated in 1 \times PBS for 5 min on a rocker at \sim 100 rpm speed. Cells were blocked with 5% normal mouse serum (Invitrogen) in 1 \times PBS for 30 min on a rocker at \sim 100 rpm. Cells were washed two times with and incubated in 1 \times PBS for 5 min on a rocker at \sim 100 rpm. Cells were then incubated in 300 nM DAPI (Invitrogen) in 1 \times PBS for 5 min in the dark on a rocker at \sim 100 rpm. Cells were washed with and incubated in 1 \times PBS for 5 min on a rocker at \sim 100 rpm. To 200 μ L of 1 \times PBS was added 60 μ L of AF488-labeled anti-p65 (Santa Cruz Biotechnologies). Cells were allowed to incubate for 2 h in the dark on a rocker at \sim 100 rpm. Cells were washed three times with and incubated in 1 \times PBS for 5 min on a rocker at \sim 100 rpm. The coverslip containing fixed, stained, adhered cells was finally placed face down on a microscope slide, sealed, and imaged using fluorescence microscopy.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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