Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information for

Near-IR mediated intracellular uncaging of NO from cell targeted hollow gold nanoparticles

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Experimental section:

Materials: All materials were purchased from Sigma Aldrich unless otherwise stated. Thiol-poly(ethylene glycol) (5 kDa) (TPEG) was purchased from Nanocs (*PG1-TH-5k*).

Synthesis of hollow gold nanoshells. The HGN were prepared according to literature procedures.[1] This was initiated by preparing a silver seed solution by heating a mixture of $AgNO_3$ (0.2 mM) and sodium citrate (0.5 mM) in water (500 mL) to 60 °C then adding a 600 µL aliquot of 1 M $NaBH_4$. After 2 h, an additional 1.5 mL aliquot of aq. $AgNO_3$ (0.1 M) and a 600 µL aliquot of aq. $NH_2OH.HCl$ (2 M) were added, and the solution was allowed to stir overnight at room temperature. The resulting silver seed nanoparticles were subjected to galvanic replacement with gold by adding a 3.8 mL volume of aq. $HAuCl_4$ solution (25 mM) in nanopure water and then heating the solution to 60 °C for 4 h. The resulting HGN were purified by a 20 kDa MWCO dialysis cassette for 12 h against against a 500 *µM citrate buffer solution at pH 5.5.*

Optical Characterization of HGN. The optical properties were characterized by UV-Vis absorption using a Tecan Infinite 200 Pro microplate reader on a 96-well flat clear bottom plastic plate (BD Biosciences). Using a Nanosight LM10HS (Nanosight, Amesbury, UK) we estimated the HGN to have an extinction coefficient of 3×10^{10} at 800 nm. Size analysis, poly dispersity analysis, and zeta potential were determined using a Malvern ZetaSizer Nano ZS (UK). To further measure the size distribution of the hollow gold nanoshells, the particles were visualized by transmission electron microscopy using a JEOL 123 TEM microscope.

Synthesis of the thiol functionalized cupferron: ammonium N-nitroso(4-mercaptomethylphenyl)-hydroxylamine (TCF). This was prepared according to a literature procedure.[2] Briefly, solid potassium thioacetate (0.63 g, 5.52 mmol) was added to a solution of 4-nitrobenzylbromide (1.0 g , 4.6 mmol) in dry tetrahydrofuran 15 mL. This mixture was stirred at 30 °C for 13 h to form 4-nitrobenzyl ethanethioate. This product was purified by removing the solvent under reduced pressure then dissolved in chloroform and washed with water using a separatory funnel. The chloroform was removed under reduced pressure to give 0.68 g of the solid 4-nitrobenzyl ethanethioate**.** ^{1}HNMR *δH(400 MHz, CD_3OD) 2.2 (s, 3H), 4.0 (s, 2H), 7.4 (d, 2H), 8.0 (d, 2H).* The TCF was synthesized from this intermediate by preparing a solution of ammonium chloride (0.039 g, 0.73 mmol) in methanol to which was added 0.14 g (0.65 mmol) of 4-nitrobenzyl ethanethioate was added for a final volume of 6 mL. Water was added to give a 5:1 MeOH :H_2O ratio and then Zn dust (0.086 g, 1.31 mmol) was added and the resulting mixture was stirred vigorously for 2 h at 0 °C. The resulting $Zn(OH)_2$ was filtered out and the solution was again cooled to 0 °C. At this stage, isobutylnitrite (73 µL, 0.58 mmol) was added and the mixture was stirred for 45 minutes at 0 °C. Excess ammonia gas was added to the headspace for 5 minutes in order to deacetylate the thiolacetate and form the ammonium salt of the thiolated cupferron. The solvent was removed under reduced pressure. HNMR: *δ_H(500 MHz, CD_3OD) 3.79 (s, 2H), 7.52 (d, J = 8 Hz, 2H), 7.92 (d, J = 8.5 Hz, 2H).*

Preparation of TPEGRP. Neuropilin-1 targeting peptide, CRPARPAR, was purchased from Lifetein with an amino terminated rhodamine for quantification and tracking studies. The thiolated polyethylene glycol derivatized with the targeting protein was prepared by conjugating the cysteine of the peptide. with Azido-PEG_3-Maleimide (Click Chemistry Tools) according to the manufacturer's protocol. In short, a 10-fold molar excess of Azido-PEG_3-maleimide was added to a 1 mM solution of the peptide in PBS, and the resulting mixture was then incubated overnight at 4°C. The azide conjugated peptides were purified by Bio-Gel P2 desalting column equilibrated with PBS and then were allowed to react with thiol-PEG_{5K}-alkyne (Nanocs) in the presence of 0.1 mM $CuSO_4$ and 5 mM ascorbic acid for 24 h. The TPEGRP was then dialyzed (3.5k Slide-A-Lyzer, Pierce) against PBS for overnight to remove the catalyst and unreacted peptide.

Preparation of TCF-HGN conjugates. These were prepared from the thiolated cupferron TCF and TPEG (or TPEGRP) by mixing a 1 mL volume of HGN solution (50 pM) in 500 µM citrate buffer pH 5.5, with a 100 µL

volume of TCF and TPEG (each 100 μM) in pH 7.4 PBS solution with 0.01% Tween20 surfactant. This was allowed to react for 13 h at 4 °C to displace the citrate surface ligands of the HGN. The sample was purified by repeatedly centrifuging down the TCF-HGN conjugates then re-suspending in pH 7.4 PBS 0.01% Tween20 buffer to remove excess thiolated cupferron, thiol PEG, and citrate. An analogous procedure was used to prepare the peptide modified conjugates but using TPEGRP instead of TPEG. Particle loading of TPEGRP was achieved by preparing a 1:1 solution of TCF-HGN-TPEGRP and an aqueous solution of KCN (0.2 M KCN, 0.002 M $K_3Fe(CN)_6$ in PBS) to etch the particle and chemically release the rhodamine labeled peptide and then measured fluorescence by a Tecan Infinite 200 Pro microplate reader to compare against a standard curve of known concentrations of peptide diluted 2 fold in KCN etch.

Nitric oxide analysis. The nitric oxide generated in photolysis cells was measured quantitatively using a calibrated GE Sievers Nitric Oxide Analyzer (NOA). Samples are withdrawn from the headspace in the photolysis cells and injected into the flowing gas stream of the NOA.

Pulsed laser irradiation. A stock solution of TCF-HGN was diluted with pH 7.4 PBS 0.01% Tween20 buffer to give working concentration of 48 pM HGNs in 100 μL samples. These samples were placed in a sealed cuvette and irradiated with Ti:sapphire femtosecond pulse laser in the UCSB Optical Characterization Facility. The excitation beam had a diameter of 5 mm and the samples were irradiated for 30 second intervals (1 kHz, 140 fs pulse length) at average powers 0.9 W cm^{-2}, 1.5 W cm^{-2}, 2.4 W cm^{-2}, and 5 W cm^{-2}. NO production was evaluated by using a gas tight syringe to sample the headspace of the photolysis cell and transport this to the NOA.

CW laser irradiation. This experiment was carried out with analogous solutions in a Y-cell through which the NO produced was carried to the NOA by a continuous flow of helium. The photoexcitation source was a 976 nm Sheaupac fiber coupled laser module with diode laser controller (LDX 3500B - 200 mA to 6 A) and laser diode temperature controller (LDT 5545B) from ILX Lightwave.

Cell culture experiments for imaging. Cells from the human prostate cell line PPC-1 and 22Rv1 were maintained in DMEM and RPMI medium 1640 with phenol red (Life Technologies) supplemented with 10% fetal bovine serum (HyClone), respectively, at 37 °C under 5% CO_2. HeLa cells were maintained in DMEM with 10% fetal bovine serum at 37°C under 5% CO_2. For cellular experiments PPC-1, 22Rv1, HeLa cells were grown on an 8 well chambered glass slide (Thermo LabTek II) at an initial seeding density of 40,000 cells per well for 24 h at 37°C in 5% CO_2 in complete media. For Co-Localization studies PPC-1 and HeLa cells were incubated with or without 10 pM of TCF-HGN-TPEGRP and 0.25 mM calcein for 2 hours. Prior to washing the cells copiously with PBS and fixing the cells with a 4% formaldehyde solution, the cells were stained with Hoescht 33342 for 5 minutes. For temperature controlled release studies using HGN, 22Rv1 cells were washed with PBS and further incubated for 1 h in the presence of a 1 mM of the nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME, Cayman Chemical) in Fluorobrite DMEM media (Life Technologies).[3] The 22Rv1 cells were then treated with 10 μM DAF-2 DA (Cayman Chemical) and a final 10 pM of TCF-HGN-TPEGRP was added to 200 μL of Fluorobrite medium in wells. In parallel a set of 22Rv1 cells treated with HGN-TPEGRP conjugates without the nitric oxide precursor was prepared as a negative control. After 2 h of incubation at 37˚C in 5% CO_2 atmosphere, the cells were rinsed with PBS prior to imaging.

Cellular Imaging of Calcein and HGN Localization. Imaging of co-localization of the particles with calcein was achieved using an Olympus 1000 Spectral Confocal microscope and an Olympus BX51 fluorescence microscope equipped with a darkfield condenser. 3D visualization and estimation of co-localization was performed using the Imaris visualization software (Bitplane, Switzerland).

Two photon imaging and in vitro NO generation. Imaging was performed using an Olympus Fluoview 1000 MPE Microscope. Living 22Rv1 cells were excited with a mode-locked Ti:sapphire tunable (690-1020

nm) femtosecond pulsed laser (100 fs pulse duration, 80 MHz repetition rate, Mai Tai HP, Newport-Spectra Physics) regulated with a modulator linked to the Fluoview software. A 25× water immersion objective with a numerical aperture of 1.05 was used. Images were collected in a 12 bit file with 512 × 512 pixels. DAF-2 DA fluorescence in cells plated on an 8 well glass slide was imaged with a 15 mW blue laser diode exciting at 473 nm raster scanning at a speed of 80 kHz. NIR photolyses of the cells containing either TCF-HGN-TPEGRP or HGN-TPEGRP conjugates were performed using the femtosecond pulsed Mai-Tai laser tuned to 800 nm at a raster scan speed of 125 kHz for 5 full-frame cycles at 4 mW. The fluorescence from the cells was imaged again with the 473 nm laser diode.

	Size	PDI	Zeta-Potential
HGN Bare	65.09	0.1793	-12.87
HGN + TCF	92.74	0.1503	-14.53
HGN + TCF + TPEG	92.96	0.1303	-14.93
HGN + TCF + TPEGRP	102.42	0.1357	-24.03

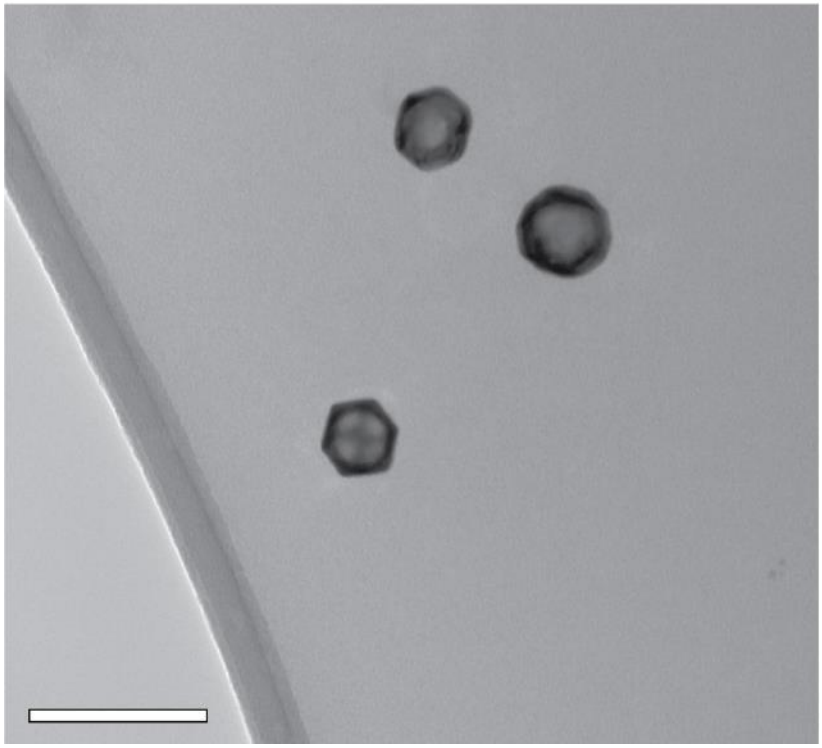

Figure S-1. (a) Dynamic light scattering, poly-dispersity, and zeta-potential analysis of HGN at different stages of assembly. (b) Additional transmission electron micrograph of bare HGN. Scale bar is 100 nm. (Note: Samples were passed through a 0.22 µm filter and sonicated for 10 seconds prior to measurements. The HGN + TCF particles had a tendency to flocculate over time; therefore, PEG was added to stabilize the constructs. For HGN + TCF samples, DLS measurements after filtration yielded the results above. However, without filtration the HGN +TCF sample were observed to have a size of 256 nm and a PDI of 0.241, while HGN + TCF + TPEG remained at ~90 nm with and without filtration.)

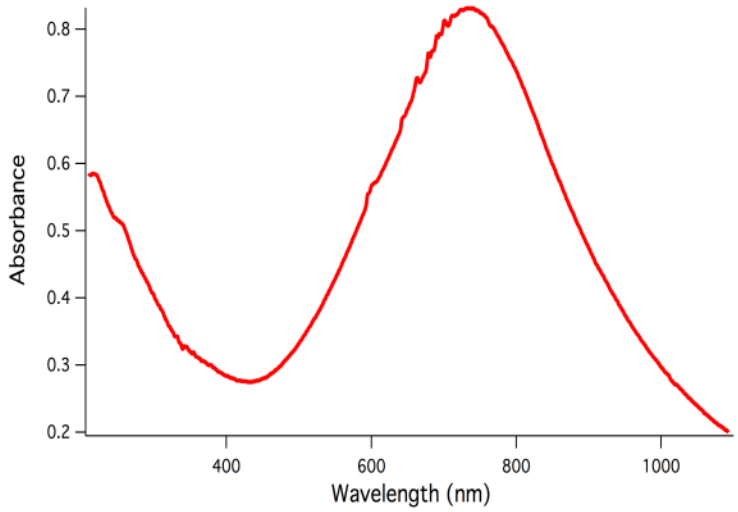

Figure S-2. Absorption spectrum of a solution of TCF-HGN-TPEG conjugates (28 pM) in PBS with 0.01% Tween20 surfactant (pH 7.4) showing the HGN surface plasmon centered at ~ 750 nm. The scattering from the HGN particulates gives a high background absorbance.

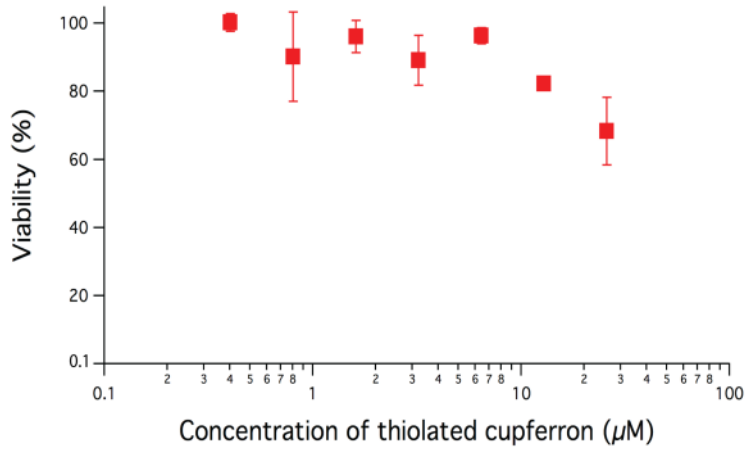

Figure S-3. Viability of PPC-1 cells after treatment with thiol cupferron (TCF). Cells were plated at 5000 cells per well and then incubated for 24 h at 37 °C. Various concentrations of the TCF were then added and the cells further incubated for 24 h at 37 °C. Cell viability was then compared to a control to which no TCF had been added. No cell death was observed in the control according to the PrestoBlue staining assay (Life Technologies), and no significant toxicity was noted at TCF concentrations <10 µM.

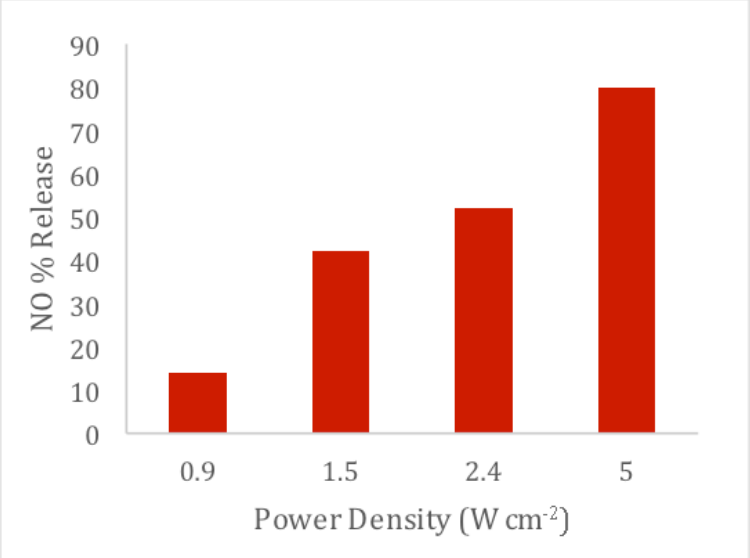

Figure S-4. Percent of NO release from TCF-HGN-TPEG conjugates upon excitation with an 800 nm pulsed laser operating at 1 KHz. Percentages were determined based on experimentally measured maximum TCF loading on the TCF-HGN-TPEG conjugates.

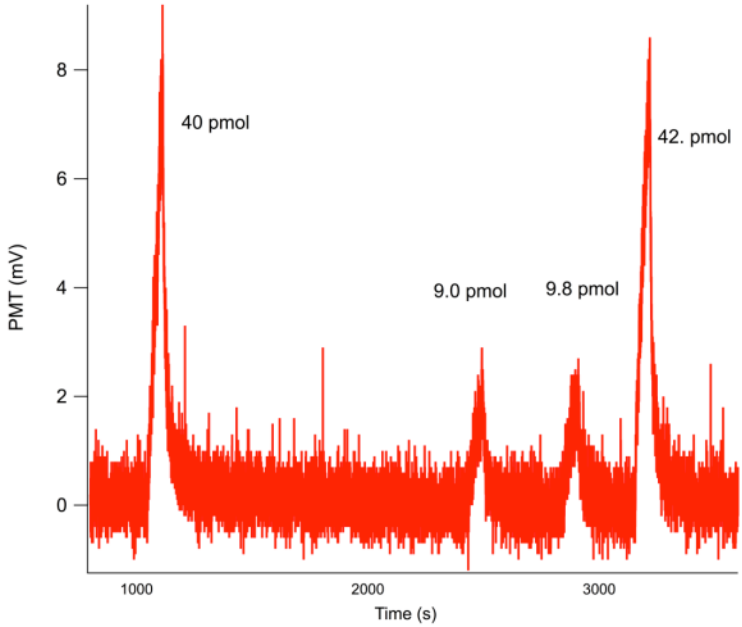

Figure S-5. NOA signals demonstrating NO release from TCF-HGN-TPEG conjugates upon excitation with a 800 nm CW laser. The irradiation time in each case was 60 s with beam intensities of 157 W cm^{-2} (first and fourth) or 78.5 W cm^{-2} (second and third) and a beam diameter 0.9 mm.

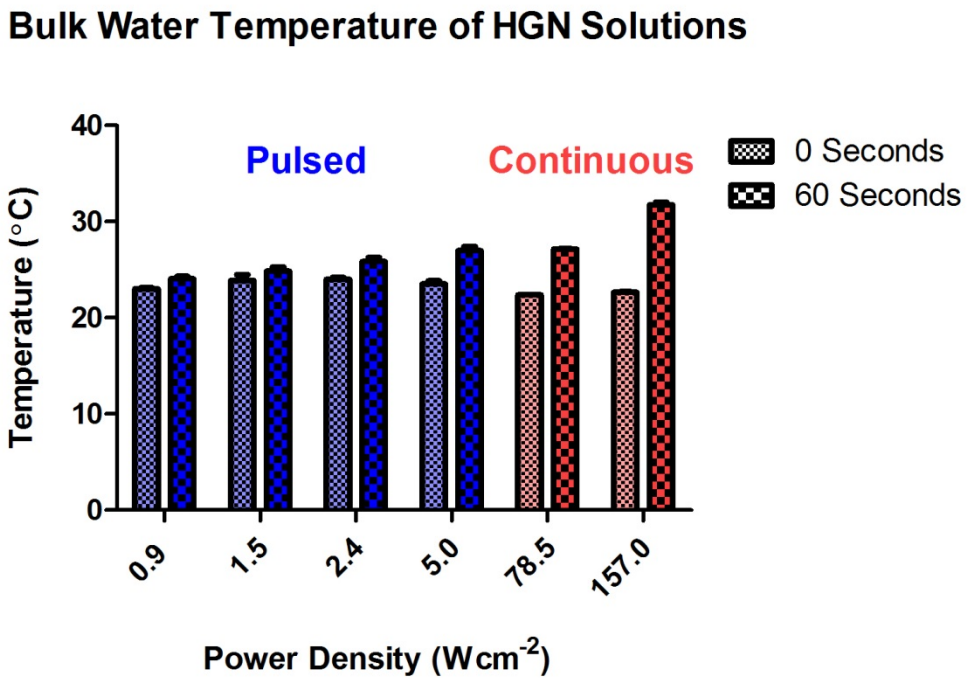

Figure S-6. Temperature rises in bulk solutions upon pulsed or CW laser photolysis. Temperature was measured by a digital temperature probe placed in the HGN solution in a micro cuvette in a position that did not obstruct the laser path. The temperature was measured before and after 60 seconds of laser irradiation at different power densities and different laser sources (refer to experimental details).

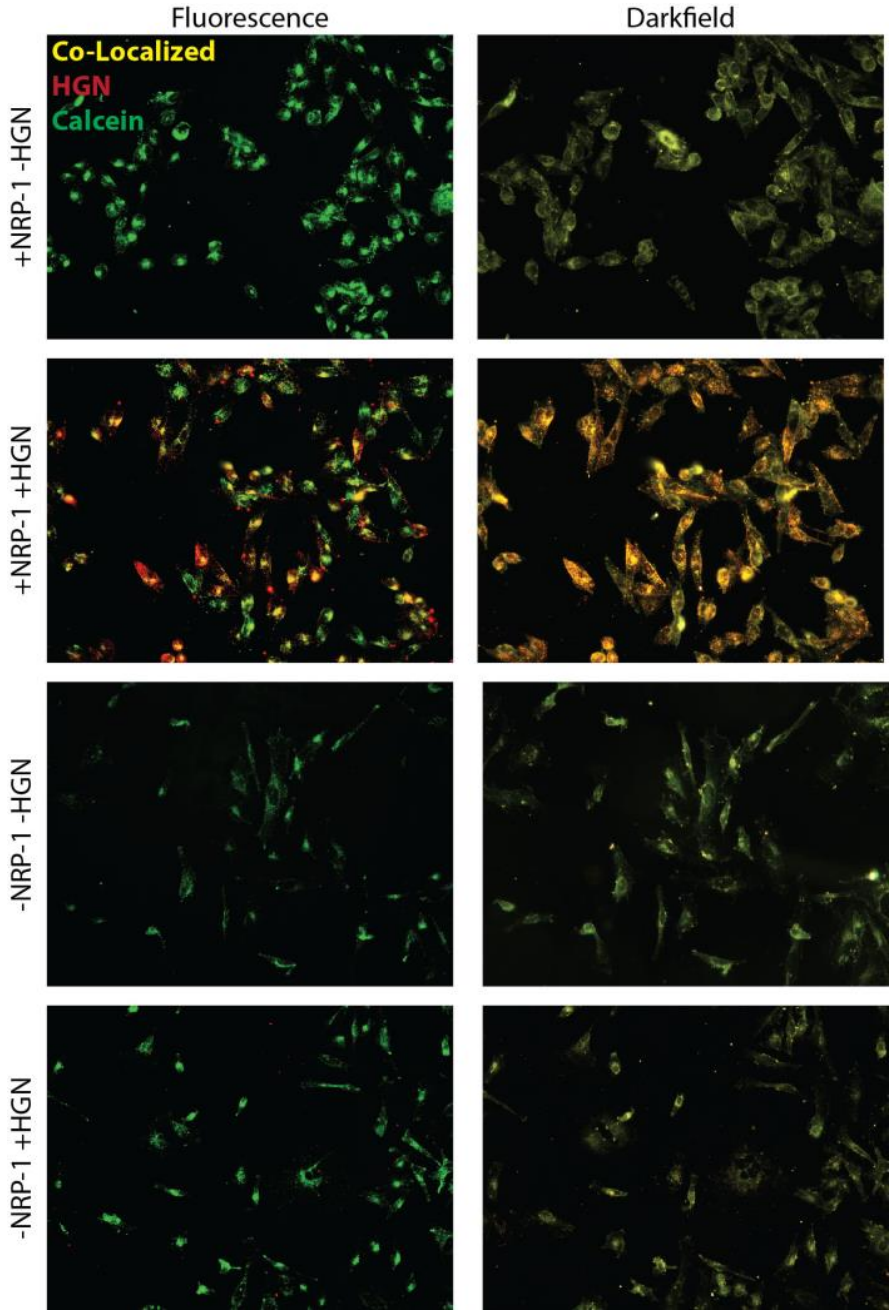

Figure S-7. *Left Panels:* Visualization of the TCF-HGN-TPEGRP conjugates co-localized with calcein (GREEN) in the endosomes of human prostatic PPC-1 cells through fluorescence microscopy of rhodamine dye conjugated to the peptide (RED). *Right Panels*: Scattering of the plasmonic particles with Dark Field Microscopy (DMF). The bright spots indicate HGNs target only those cells expressing Neuropilin-1 (+NRP-1 +HGN). HeLa cells do not show localization of HGN in endosomes (-NRP-1 +HGN). Samples without HGN (+/-NRP-1 –HGN) are presented as reference to the natural scattering observed in the cells.
(*Note:* Attempts to image NO release in the PPC-1 cells using DAF-2 DA proved inconclusive owing to high background fluorescence, and for this reason, those experiments were done with the human prostate cell line 22Rv1.)

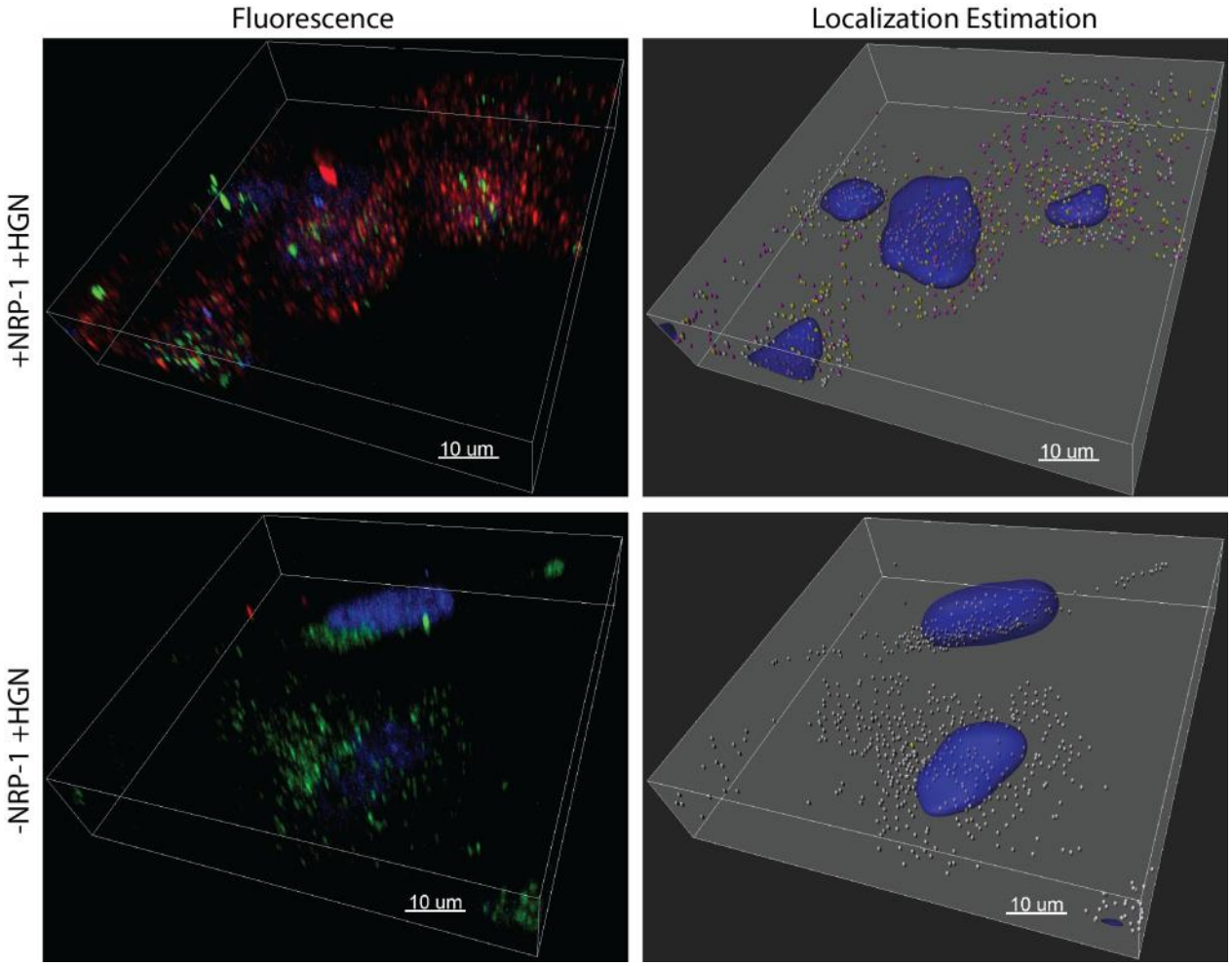

Figure S-8. Depth analysis of particle localization performed by confocal microscopy. *Left Panels*: Fluorescence imaging of markers for calcein (GREEN), HGN (RED), and nuclei (BLUE). *Right Panels*: 3D visualization of fluorescence imaging performed by the Imaris software to estimate co-localization within 1 µm of green and red signals. *Top Panels*: PPC-1 cells overexpressing Neuropili-1 show fluorescence signals from both calcein and HGN, indicating that HGN labeled with the C-end rule peptide, RPARPAR, are internalized by receptor mediated endocytosis. Estimation of the co-localization shows that there is a number of signals from both calcein (WHITE) and HGN (MAGENTA) as discerned from the YELLOW spots. *Bottom Panels*: HeLa cells that lack the Neuropilin-1 receptor only show markers for endocytosis but do not demonstrate internalization of HGN. Furthermore, there is little to no indication of co-localizaiton of fluorescence in the 3D visualzaiton. Scale bar is 10 µm.

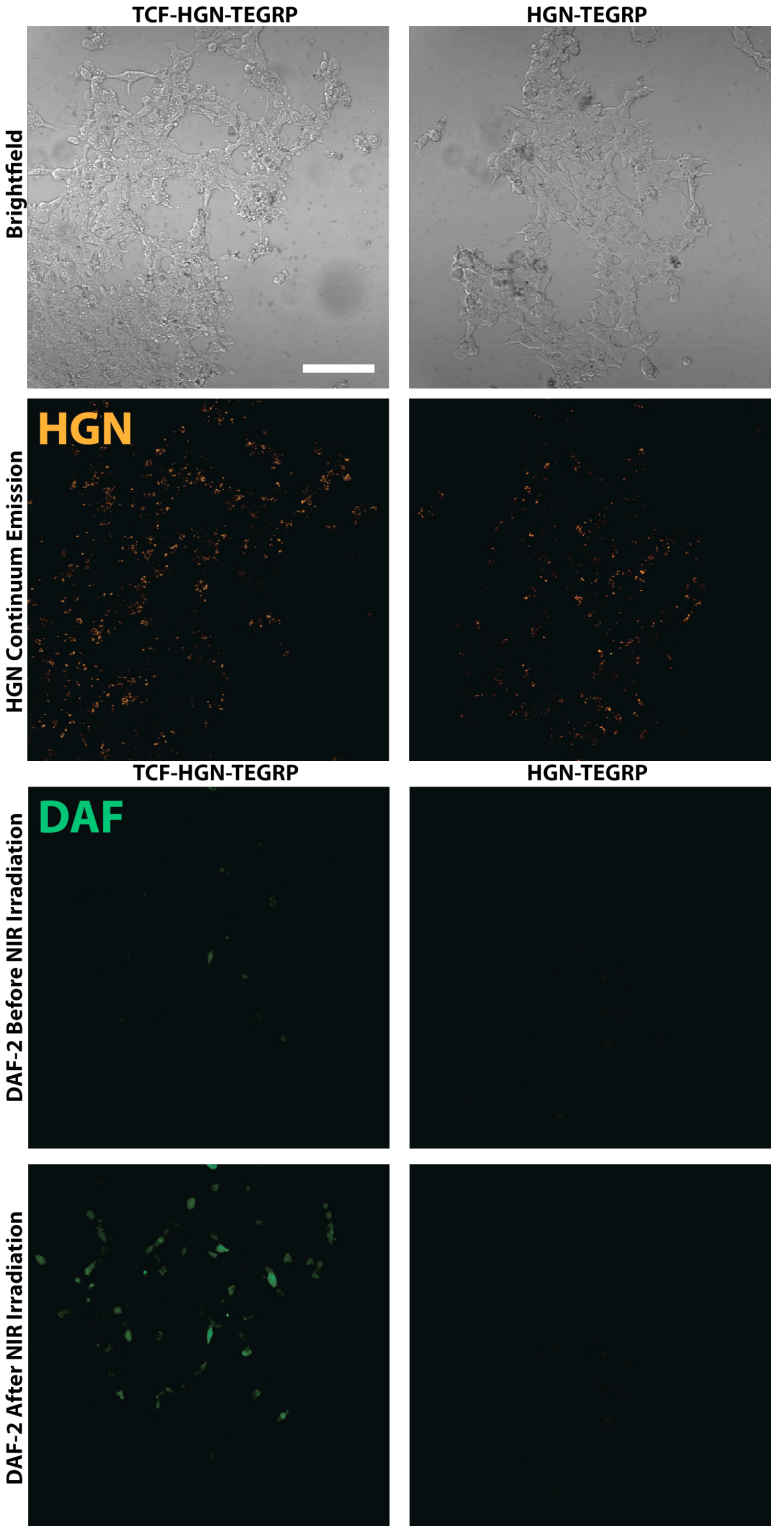

Figure S-9. Expansion of Figure 4. Spatio-temporal controlled release of NO from HGN in 22Rv1 cells treated with TCF-HGN-TPEGRP (left) and HGN-TPEGRP (right). NO production was characterized by the detection of the DAF-2 DA fluorescent reporter. *First row:* brightfield image of the 22Rv1. *Second row*: continuum emission of HGN detected upon laser irradiation (orange). *Third and fourth row*: DAF-2 DA fluorescence (green) before and after 800 nm laser irradiation of the HGN. Scale bar is 100 µm.

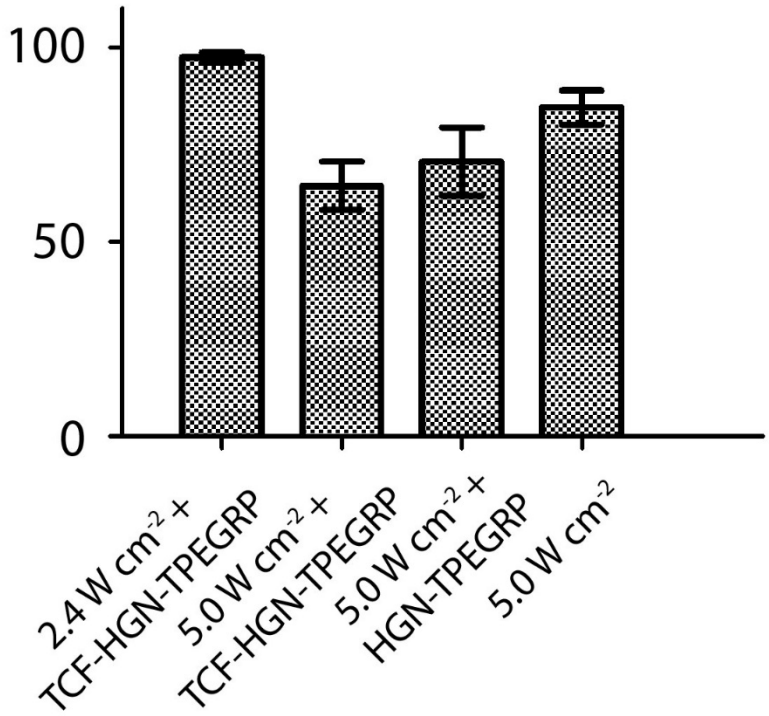

Figure S-10. Cellular viability after 30 seconds laser exposure in cells treated with the HGN-TPEGRP with and without TCF relative to a cell only control. Only at high laser power irradiation (5.0 W cm^{-2}) is viability slightly compromised, ~20 to 40% loss, as observed by bare cells exposed to laser and cells carrying the TCF-HGN-TPEGRP construct and irradiated, respectively.

References

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