Supporting Information

Continuous Spectroscopic Measurements of Photo-stimulated Release of Molecules by Nanomachines in a Single Living Cell

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1 Characterizations of Azo-NP (Table S1, Fig. S1-S5)

Table S1: Dynamic Light Scattering (DLS) measurements. The size of Azo-NP was determined by DLS using a ZetaPALS instrument (Brookhaven Instrument Corporation), with an excitation source of 632.8 nm HeNe laser [90° to the incident beam and at 25 °C]. Tables S1(a) and S1(b) report the size of Azo-NP in water and phosphate buffer saline (PBS) medium respectively. Prior to measurements, Azo-NP was sonicated in the respective solutions for 15 minutes. As shown, the size of Azo-NP increases in PBS medium, implying that the presence of ionic salts facilitates aggregation of the particles. (Center for Environmental Implications of Nanotechnology (CEIN) at California Nanosystem Institute (CNSI))

Table S1(a): Azo-NP in distilled water

Run	Diameter
1	288.4 nm
2	277.6 nm
3	263.4 nm

Table S1(b): Azo-NP in PBS buffer

Run	Diameter
1	394.8 nm
2	421.9 nm
3	512.0 nm

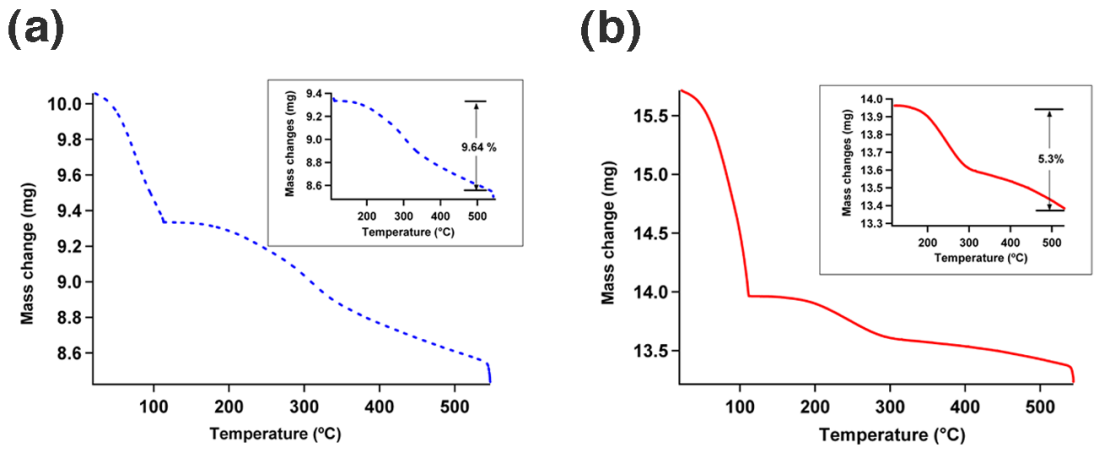

Figure S1: Thermogravimetric Analysis (TGA). Thermogravimetric measurements of Azo-NP and unmodified MCM-41 silica nanoparticles (np) were conducted on a Perkin Elmer Pyris 6 TGA in air. Heating rate was 25°C/min from 20°C to 120°C, and 10°C/min from 120°C to 600°C. **(a)** Azo-NP exhibits a weight loss of approximately 9.64% while **(b)** MCM-41 silica np shows a weight loss of 5.3% over the temperature range of $120 - 600$°C. The weight loss from MCM-41 silica np can be attributed to the loss of strongly adsorbed water molecules inside the pores as well as dehydration of silanol units. Subtracting 5.3% from 9.64%, a weight loss of 4.34% is associated with the amount of azo-linker molecules that have been successfully co-condensed into Azo-NP.

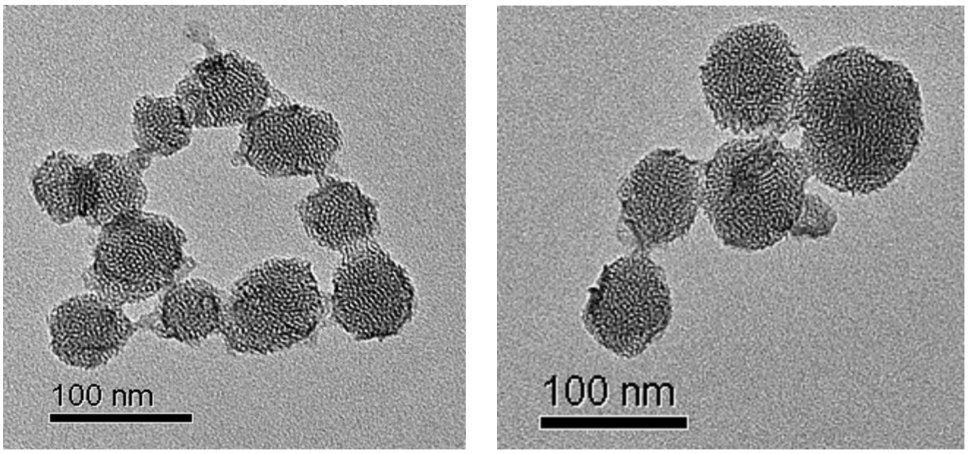

Figure S2: Transmission Electron Microscopy (TEM). TEM images show that individual Azo-NP has a mean diameter of 65 nm, with an average diameter of 300 nm when they cluster together. These micrographs were obtained using a JEM1200-EX (JEOL) instrument operating at an acceleration voltage of 80 kV in the Electron Imaging Center for Nanomachines (EICN) at CNSI.

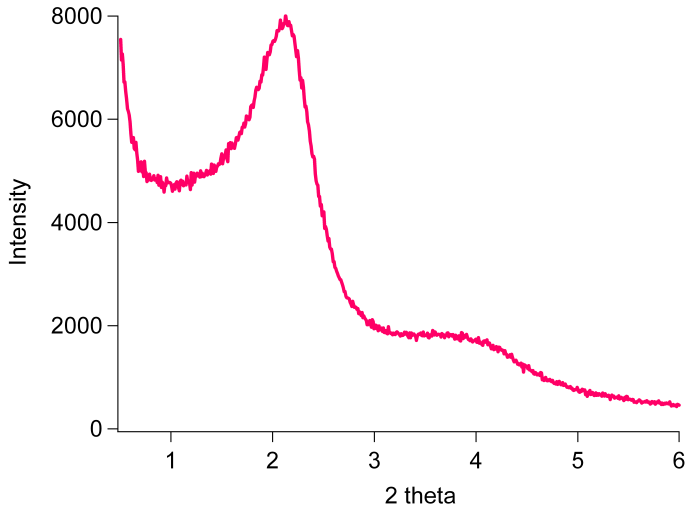

Figure S3: Powder X-Ray Diffraction (PXRD). Mesoporosities of Azo-NP were verified using low angle PXRD. The particles exhibit 2θ peaks at 2.2° and 4.0° that are characteristics of the (100) and (110) planes respectively. From the 2θ peak at 2.2°, lattice spacing is determined to be ∼3.5 nm. PXRD patterns were collected using Philips X'Pert Pro diffractometer equipped with CuKα radiation.

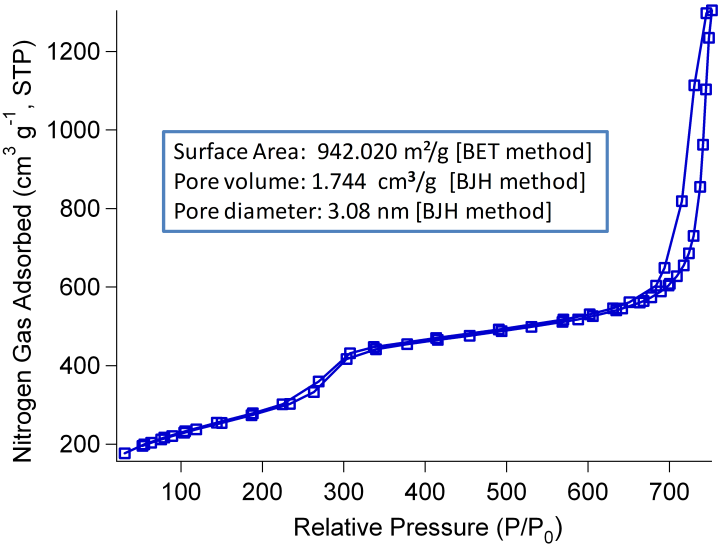

Figure S4: Nitrogen Adsorption/Desoprtion: Pore size, pore volume and surface area. Applying the BET (Brunauer, Emmet, and Teller) model to the data set, surface area of Azo-NP was determined to be 942 m^2/g. Pore volume and pore diameter were evaluated by the BJH (Barrett, Joyner and Halenda) method to be 1.74 cm^3/g and 3.08 nm respectively. N_2 adsorption-desorption isotherms were obtained at 77 K on a Quantachrome analyzer in CEIN at CNSI.

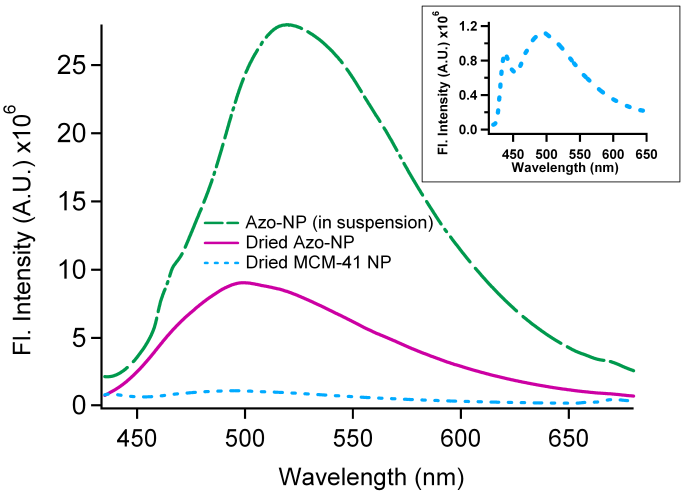

Figure S5: Luminescence of Azo-NP and MCM-41. Emission of the particles was measured by an Acton SpectraPro 2300i CCD detector using a front face geometrical configuration with excitation at 408 nm (30 mW) [CUBE 405-50C diode laser, Coherent]. The intense fluorescence exhibits from Azo-NP supports the notion that when azobenzene molecules are confined in a nanometer-scale environment, fluorescence can be observed. As shown, moistened Azo-NP is more fluorescent than its dried form, highlighting the impact of solvent on the luminescence (inset shows an expanded spectrum of MCM-41).

2 Photo-chemical properties of Azo-NP (Fig. S6-S8)

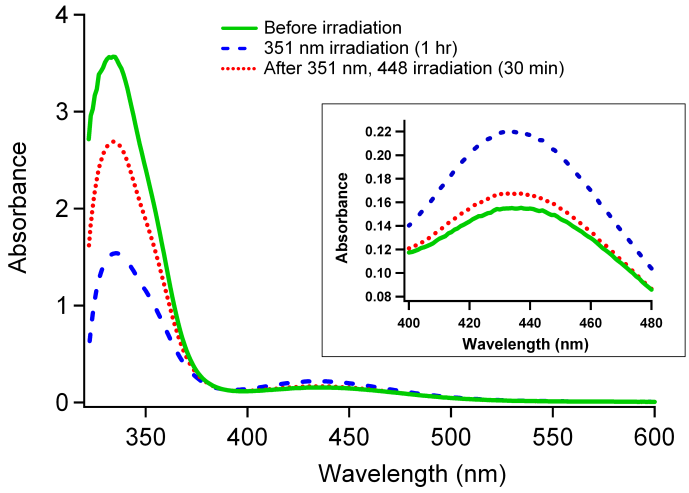

Figure S6: Absorption profile of Azo-linker. Azo-linker's capability of undergoing reversible photo-isomerizations was determined by measuring the changes of its two absorption bands in the UV-visible region. After irradiating the azo-linker solution (acetone) at 351 nm for 1 hour to reach a *cis*-rich photo-stationary state, a decrease in absorbance at 330 nm [*trans* isomer] accompanied with an increase in 430 nm were observed (blue, dashed trace). When the UV-exposed solution was irradiated at 448 nm for 30 min (red, dotted trace), the reversed trend was observed.

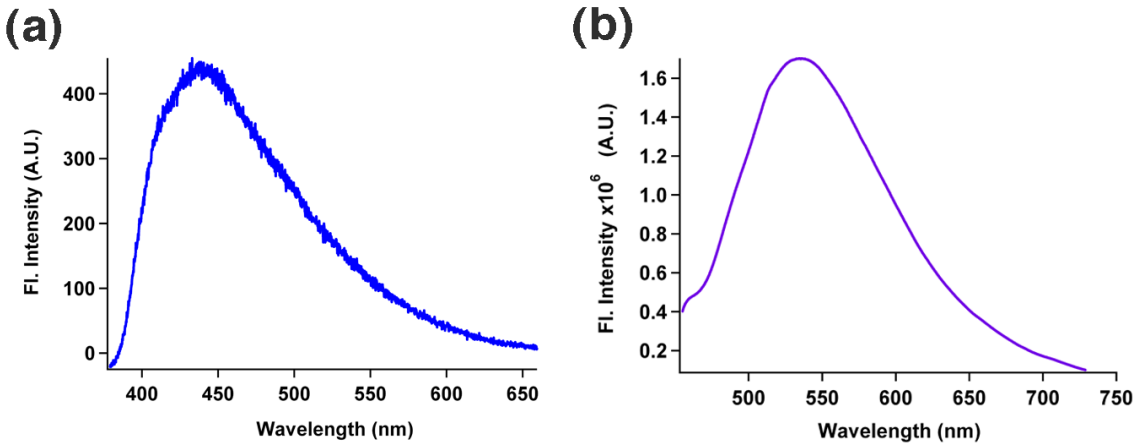

Figure S7: Fluorescence emission spectra of Azo-NP. Fluorescence emission spectra of Azo-NP were collected by an Acton SpectraPro 2300i CCD detector at a front face geometrical configuration with excitation at **a)** 351 nm (Innova 300C argon ion laser, Coherent) and **(b)** 448 nm (CUBE 445-40C diode laser, Coherent).

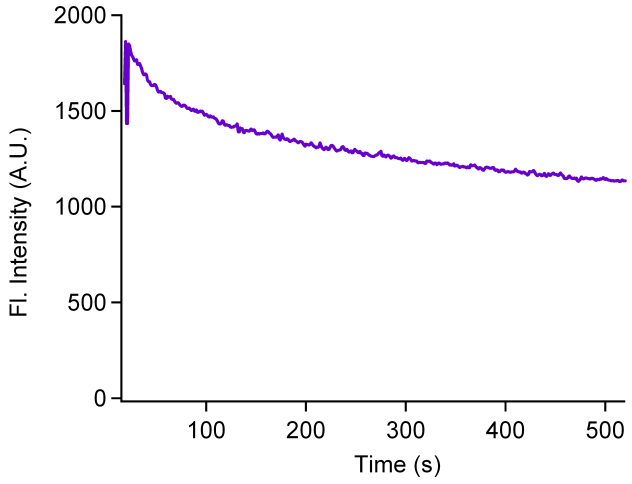

Figure S8: Efficiency of the isomerization process within pore channels. Enhanced fluorescence emission is often observed in conjunction with a slower rate of photo-isomerization, suggesting that confinement of azobenzene molecules in a restrictive environment hampers their non-radiative process. Continuous excitation of Azo-NP at 351 nm did not lead to an increase in fluorescence intensity at its emission maximum (440 nm), which suggests the *cis-trans* motion was not impeded.

3 Control studies in solution (Fig. S9-S11)

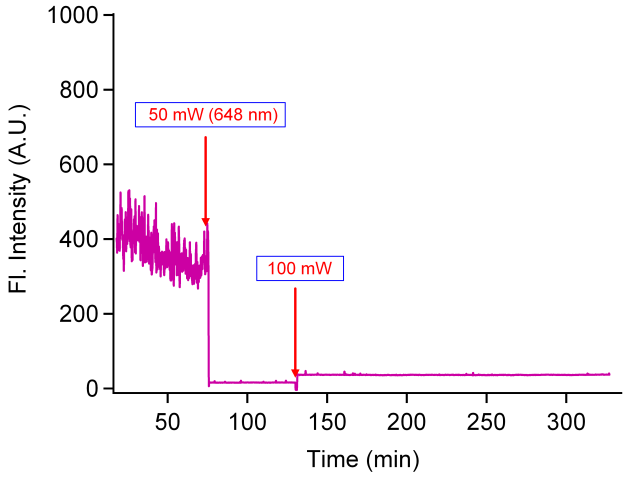

Figure S9: Control study in solution: laser heating. To verify that activation of Azo-NP is not a result of laser heating, 648 nm light (a wavelength where azobenzene molecules do not absorb) was used to excite these particles. The spectroscopic setup and probe beam (514 nm) were the same as shown in figure 1b. Once the baseline was established, Azo-NP (loaded with PI) was irradiated with 648 nm laser beam, initially at 50 mW and later at 100 mW. Intensity changes at PI's emission maxima (630 to 660 nm) were plotted as a function of time.

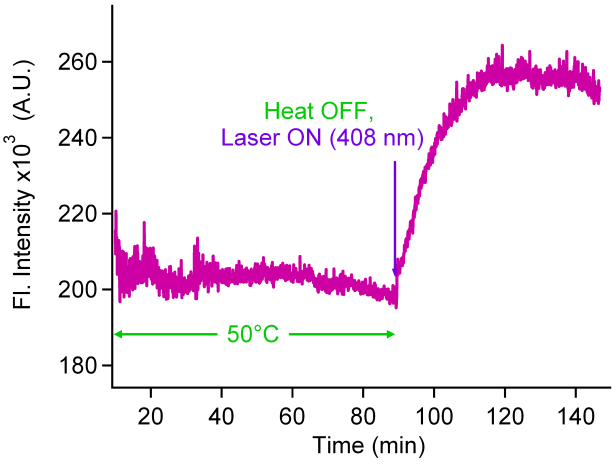

Figure S10: Control study in solution: thermal heating. To ensure that thermal heating is not an activation mechanism, fluorescence intensity changes at PI's emission maxima was monitored for 90 minutes in 50°C aqueous solution (using the same setup and probe beam as shown in figure 1b). As shown, no changes in intensity were observed at this setting. After the heat was turned off, the beam that excites Azo-NP (408 nm, 20 mW) was turned on and fluorescence intensities increased.

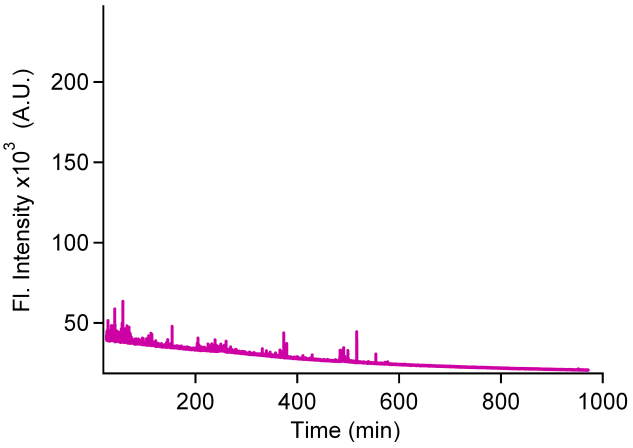

Figure S11: Control study in solution: stability of Azo-NP. Azo-NP was placed on a corner of the cuvette, and the cuvette was filled with solution. With the solution being gently stirred (but leaving the particles undisturbed) and in the absence of any excitation beam, fluorescence changes in solution were continuously monitored for more than 16 hours. (Same setup and probe beam were used as shown in figure 1b)

4 Confocal micrographs of Azo-NP uptaken by cancer cells (Fig. S12)

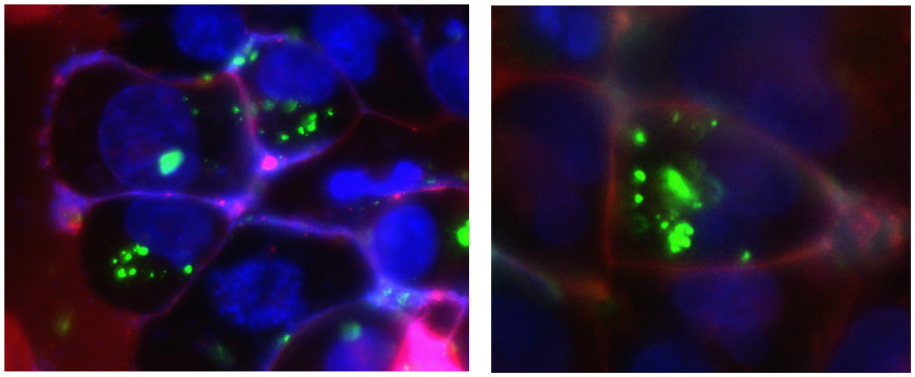

Figure S12: Internalization of Azo-NP by cancer cells. MiaPaca-2 pancreatic cancer cells ($\sim 40 - 60\mu$m in size) were incubated in the dark with Azo-NP (10μg/mL). Prior to taking these images, the cells were washed with medium and PBS buffer to remove any particles that did not enter the cells. The cells were then stained with DAPI for nuclei (blue fluorescence), WGA for plasma membrane (red fluorescence). The green fluorescence shown is due to the intrinsic emission from Azo-NP. (Left, 40 X; right, 63 X)

5 Background fluorescence (Fig. S13-S14)

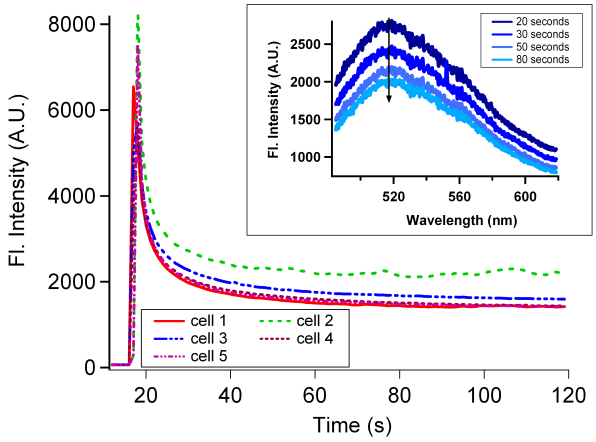

Figure S13: Autofluorescence from a single cancer cell. A single cancer cell excited at 408 nm (30 mW) shows an emission peak centered at ∼520 nm. Changes in fluorescence intensities at its emission maxima (500 - 530 nm) were monitored for 5 randomly-chosen viable cancer cells. As shown, intensities attenuated for all 5 cells after 30 s of irradiation. (Inset) Selected emission spectra of "cell 3" demonstrate the intensity decrease [Note: cell viability was checked after each trial to ascertain the decrease in intensity was not a sign of cell death].

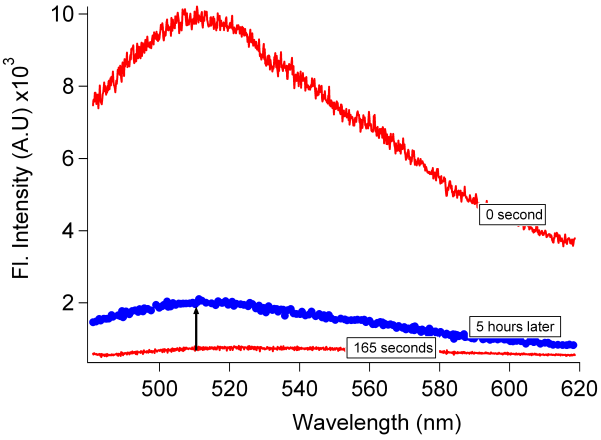

Figure S14: Photostationary state. A droplet of Azo-NP in water was blotted on a microscope slide. 408 nm laser beam (30 mW) was focused onto a small cluster of Azo-NP using the epifluorescence instrument. Emission spectra were collected for 180 s. The laser was turned on again after a 5-hour hiatus, and another emission spectrum was acquired. As the figure illustrates, luminescence intensities of Azo-NP taper off after 165 s of irradiation. In the absence of any light, $\sim\frac{1}{5}$ of its original intensities were restored after 5 hours.

6 Control studies *in vitro* (Fig. S15-S16)

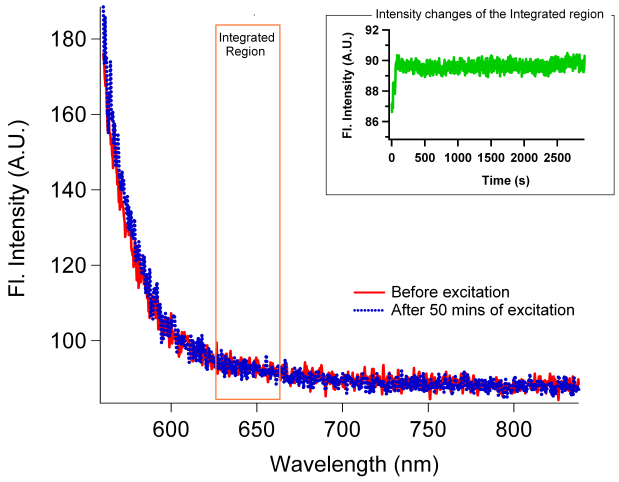

Figure S15: *in vitro* control study. Cancer cells incubated with Azo-NP, loaded with PI molecules, were illuminated with 514 nm light (30 mW) for 50 minutes using the epifluorescence instrument. Based on the emission spectra of the before (red) and after (blue) excitation, no release of PI was observed (inset: Intensity changes at PI's emission maxima were plotted as a function of time).

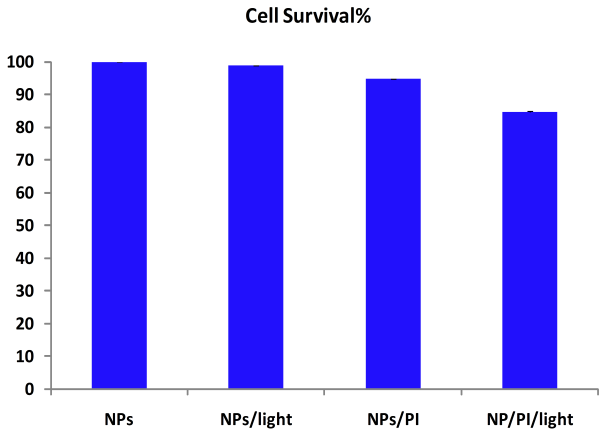

Figure S16: Cell viability. Cytotoxicity assays were performed on four sets of cells using a cell-counting kit purchased from Dojindo Molecular Technologies. The columns, from left to right, represent: cells with Azo-NP, cells with Azo-NP (no PI) subject to laser exposure (408 nm, 30 mW), cells with PI-loaded Azo-NP, and cells with PI-loaded Azo-NP subject to laser exposure. As shown, our experimental conditions did not significantly decrease cell viability.

7 Methods

7.1 Azo-NP synthesis.

Azo-NPs were synthesized by co-condensing azo-linker molecules to surfactant-templated mesoporous silica nanoparticles. The azo-linker was made by refluxing isocyanatopropyltriethoxysilane [7.57 X 10^{-4} M] with 4-hydroxyazobenzene [3.78 X10^{-4} M] in 5 mL of acetone for 4 hours under inert atmosphere (**$\sim$2.19 μmol/mg of particles**). In another round-bottom flask, cetyltrimethylammonium bromide [1.38 X 10^{-3} M] was dissolved in 250 mL of distilled water along with 1.75 mL of 2 M NaOH. The solution was heated to 80 °C before adding the azo-linker. The reaction mixture was then stirred for an additional 15 minutes before adding 2.5 mL of tetraethylorthosilicate. The solution was allowed to stir for another 2.5 hours at 80 °C. Azo-NPs were isolated by centrifuging and washed with ample amounts of water and methanol. The surfactant template was then extracted by refluxing the particles in acidic medium under inert atmosphere overnight. Characterizations of Azo-NP are reported in supplementary information.

7.2 Experimental setup for light-activated release in solution.

Azo-NP's photo-responsiveness was tested by measuring the release of PI molecules that were stored inside the pores. Release of PI was measured by monitoring spectral emission changes in solution using luminescence spectroscopy. After Azo-NP was loaded with PI cargo molecules (loading procedure was similar to that reported elsewhere[1, 2]), the particles were placed in a corner of a cuvette. Water was slowly and carefully added to prevent disturbing the particles. While constantly stirring (stir bar was placed at the opposite corner from the particles), a probe beam (514 nm, Innova 300C argon ion laser, Coherent) orthogonal to the detector was used to continuously irradiate the supernatant and excite the cargo molecules as they were released into the solution. Spectral changes in the solution were monitored by an Acton SpectraPro 2300i charge-coupled device (CCD) detector. The CCD detector collects sequential emission spectra, once per second, and generates a three-dimensional plot of luminescence intensity, wavelength and time. Once the fluorescence intensity baseline was established, a pump beam – 408 nm (CUBE 405-50C diode laser, Coherent) was introduced to directly irradiate Azo-NP.

7.3 *In vitro* observation using the epifluorescence instrument.

The epifluorescence instrument consists of an fluorescence microscope with laser excitation and spectroscopic detection. A laser beam (beam size $\sim 40\mu m$) was introduced to a Nikon Labophot-2 fluorescence microscope by removal of the rear lamphouse component. This modification was accompanied by the attachment of a fiber optic to the trinocular head through use of adapters to couple the emission to the spectrometer. The intracellular spectroscopic measurement was done by carefully directing a laser beam into the microscope, where it passes through a dichroic mirror and a 40x objective lens and focusing the beam onto the specimen tightly to allow for selective excitation. The light emitted from the sample then exits the microscope through the fiber optic into an Acton SpectraPro 2300i CCD.

MIA PaCa-2 cells were seeded in a 8-chamber plate, and incubated with Azo-NP (loaded with PI molecules) in cell culture medium overnight (Leibovitz's L-15 medium (Cellgro) supplemented with 10% fetal calf serum (Sigma, MO), 2% L-glutamine, 1% penicillin and 1% streptomycin). Prior to the experiment, cells were washed with PBS buffer. Then, a small amount of cell culture medium was coated on top of the adhered cell layer and finally, a cover slide was placed on top of the cells. The cells were kept at room temperature throughout the spectroscopic measurement.

7.4 Cytotoxicity Assay.

MIA PaCa-2 cells were seeded in a 96-well plates (approx. 5000 cells/well) and incubated with (and without) Azo-NP in fresh culture medium at 37°C in a 5% CO_2/95% air atmosphere for 24 hours. After incubation, cytotoxicity assays were performed to investigate the effects of Azo-NP, PI and laser irradiation on cell viability. Four sets of cells were examined. The first set consists of MIA PaCa-2 cancer cells with Azo-NP (no PI). The second set consists of cells exposing to Azo-NPs (no PI) and exposing to 408 nm light (30 mW) for 15 minutes. The third set consists of cells incubating with PI-loaded Azo-NP. The fourth set consists of cells incubated with PI-loaded Azo-NP and exposing to 408 nm (30 mW) light for 15 minutes. After various treatments (as described), cells were kept in the incubator for an additional 48 hours prior to washing with PBS and re-incubating with 10% WST-8 solution for another 2 hours. Cytotoxicity was examined using a cell-counting kit purchased from Dojindo Molecular Technologies Inc. The absorbance of each well was measured at 450 nm with a plate reader. Using a previously prepared calibration curve, absorbance of each well was correlated with the number of viable cells in the medium.

7.5 Spinning Disk Confocal Microscopy.

A Leica DM6000B upright microscope fitted with a Yokogawa CSU-X1 spinning disk and Photron high-speed camera was used to spatially monitor and continuously image intensity changes of a cell nucleus as Azo-NP was being excited. Through the use of two laser sources: one for azobenzene excitation (405 nm) and another for imaging (488 nm), background fluorescence was spectrally separated. The two beams were synchronized in the following way: for every twenty frames of 405 nm excitation (9.5 mW diode), a single frame of 488 nm (20 mW diode) was acquired. [CNSI Advanced Light Microscopy/Spectroscopy Facility].

References

[1] J. Lu, E. Choi, F. Tamanoi and J. I. Zink, *Small*, 2008, **4**, 421-426.

[2] S. Angelos, E. Choi, F. Vögtle, L. De Cola and J. I. Zink, *J. Phys. Chem. C*, 2007, **111**, 6589-6592.