Electronic Supplementary Material (ESI) for Molecular BioSystems

Supplementary Information

Patterning of Cells Through Patterning of Biology

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Materials and Methods

Reagents:
siRNAs were obtained from Integrated DNA Technologies. 1-(4,5-dimethoxy-2-nitrophenyl) diazoethane generation kit, oligofectamine and cell culture media were obtained from Invitrogen. Acetonitrile, ammonium acetate and triethylamine were obtained from Fisher Scientific. Dimethyl sulfoxide (DMSO) and ethanol were obtained from Acros Organics. RNase free water was obtained from MP Biomedicals. Glycogen was obtained from USB. 24-well black plates were obtained from Krystal. Calcein AM was obtained from PromoKine. Magic Dark was obtained from Rayzist.

siRNA caging with DMNPE-diazo:
A solution of 4, 5-dimethoxy-2-nitroacetophenone hydrazone (2.5 mg, 0.011 mmol) was prepared in DMSO (250 μL). The reaction was initiated with the addition of MnO_2 (10 mg, 0.115 mmol) and was agitated gently at room temperature for 45 min. The suspension was then filtered through celite to remove the MnO_2. A volume of this filtrate (204 μL, 12.5 mM) was added to siRNA (408 μL, 25 μM) and agitated at room temperature for 24 h, protected from light. Ammonium acetate (306 μL, 10 M) added to caged siRNA and agitated for 10 min at room temperature. This was followed by precipitation using glycogen (5 μL of a 20 mg/ml solution) and ethanol (2.3 ml).

HPLC analysis:
Precipitated caged siRNA duplexes were dissolved in RNase free water (90 μL) and purified with reverse phase HPLC. A Varian Microsorb C8 column (250 x 4.6 mm) was used. The mobile phase used for A was 0.1 M triethylammonium acetate buffer (pH 7) and B was 0.1 M triethylammonium acetate buffer in 50% acetonitrile. The gradient system used was 0% to 10% B from 0 to 15 min, 10% to 40% B from 15 to 25 min, 40 % to 100% B from 25 to 50 min and then maintained at 100% B until 60 min, all at 1 mL/min. All fractions were collected and freeze dried. The dried fractions were redissolved in RNase free water and analyzed by analytical HPLC and ESI mass spectrometry.

ESI mass spectrometry:
HPLC purified caged siRNA was dissolved in a mixture (100 μL) containing 50% RNase free water, 49% acetonitirile and 1% triethylamine to achieve a final concentration of 6 μM. Analysis was accomplished using Q Trap mass spectrometer (ABI) in the negative ion mode.[1]

Culture, transfection and irradiation of cells:
HeLa cells were obtained from the American Type Culture Collection (ATCC). A day before transfection, cells were plated at 5% confluency in a 24-well black plate using 500 μL of antibiotic-free DMEM supplemented with 10% FBS. The transfection mixture was prepared by combining 53 μL of diluted siRNA solution (3 μL of a 2.97 μM solution of siRNA in 50 μL of Opti-MEM) and 15 μL of diluted oligofectamine solution (2.5 μL of oligofectamine in 12.5 μL of Opti-MEM). The transfection mixture was incubated for 20 min at room temperature and then added to each well. From the experiment in figure 4 onwards, we used a modified method to insure homogeneity of the transfection solution. The transfection mixture was incubated for 20 min at room temperature and then mixed with 500 μl of fresh antibiotic-free DMEM supplemented with 10% FBS. The medium in each well was then replace with this transfection mixture (568 μl). The transfection mixture was removed from the wells after 7.5 h and the cells were washed with Opti-MEM (500 μL).

For the whole well irradiation experiment, the culture plate was divided equally in two parts. One half was irradiated and the other was protected from light using aluminum foil. A Blak-Ray UV lamp (Model XX-15L, 30 W) was used to irradiate the bottom of plate for 10 min from a distance of 10 cm. The media was then replaced with 1 mL of antibiotic-free DMEM supplemented with 10% FBS. Five days post incubation, the media was replaced with fresh media (750 μL). After 24 h the cells were stained with calcein AM (75 μL, 25 μM). Cells were incubated for 45 min and then washed with PBS (500 μL, 2 times). Fluorescence microscopy was performed in order to visualize and quantitate them respectively. Excitation and emission wavelengths of 485 and 535 nm respectively were used. The signal was quantitated using Photoshop.

For the patterning experiment, we used masks that were laser printed on Highland-903 transparencies. Transparencies were treated with "Magic Dark" 2 to 3 times to reduce the imperfections in the toner. Three types of mask were used: clear mask, 100% dark mask and a mask with a dark circle that was 20% width of entire well. The masks were glued to the bottom of the well plate. Cell culture experiments were performed using the masked well plate in an identical manner as described for whole well condition above, with an exception of increase in UV irradiation to 20 min. This was to compensate for the absorbance of the transparency film. Fluorescence microscopy was performed at the conclusion of the experiment.

For the time based photolysis study, we used strips of opaque labeling tape to protect the cells from light. The tape strips were attached to the bottom of all the wells. The plate was irradiated from the bottom using the previously described light source. Cells were exposed to varying amounts of light by removing the tape strips at 2 min intervals. Each tape was removed after 2 min of UV irradiation from the bottom leading to cells that were UV exposed for 2, 4, 6, 8 and 10 min. Cell culture experiments were performed in similar manner as described above for whole well condition. Cell images were taken using fluorescence microscope and quantification was done using Photoshop.

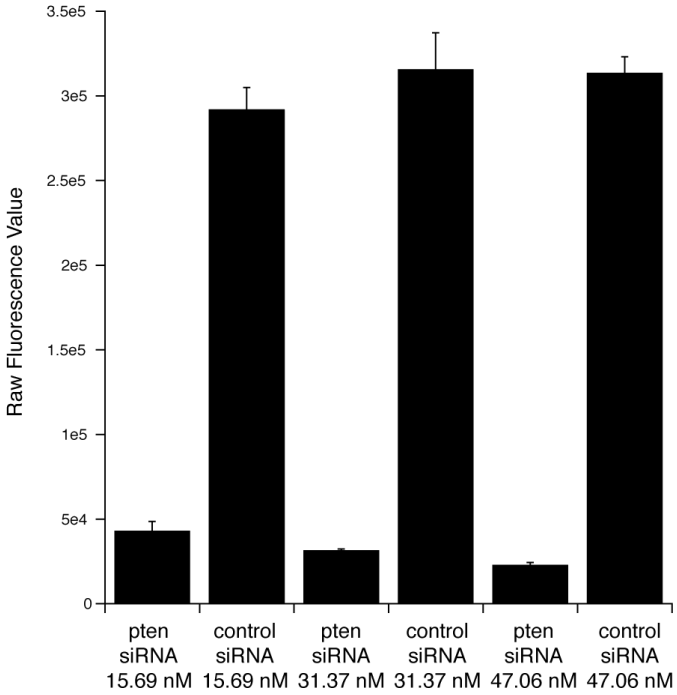
Microscopy:
Fluorescence microscopy was performed on a Nikon Eclipse TE-300 inverted epi-flourescence microscope. A 1X objective lens and 4s exposure time were used. Seven images per well were taken. All images were stitched into one complete well image using "photomerge" in Photoshop.

Fluorescent signal quantitation of well images:
Analysis of the well images was performed using Adobe Photoshop. The mean background intensity and mean intensity of the well images were determined. Then the corrected intensity was calculated by subtracting background intensity from the mean intensity. Overall signal intensity of the well images was calculated by multiplying the corrected intensity with the total number of pixels in the well image. Average of three replicates was reported.

For the time based photolysis study, the averaged signal intensity of the images at different irradiation times were normalized to the averaged signal intensity at 0 min irradiation time. This normalized signal intensity was plotted against the irradiation time.

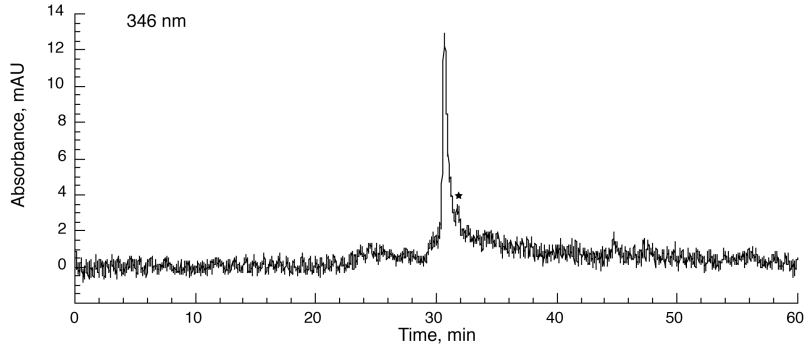
Supplementary method for the determination of the effect of varying concentration of pten and control siRNA on the number of attached cells:

A day before transfection, HeLa cells were plated at 5% confluency in a 24-well black well cell culture plate using 500 μL of antibiotic-free DMEM supplemented with 10% FBS. The transfection mixtures were prepared by combining 53 μL of diluted siRNA solution and 15 μL of diluted oligofectamine solution (2.5 μL of oligofectamine in 12.5 μL of Opti-MEM). Diluted siRNA solutions used in preparing transfection mixtures were made separately for different wells. (a) For 15.69 nM siRNA concentration wells, 3 μL of a 2.97 μM solution of siRNA was diluted in 50 μL of Opti-MEM, (b) For 31.37 nM siRNA concentration wells, 3 μL of a 5.94 μM solution of siRNA was diluted in 50 μL of Opti-MEM and (c) For 47.06 nM siRNA concentration wells, 3 μL of a 8.91 μM solution of siRNA was diluted in 50 μL of Opti-MEM. The transfection mixture was incubated for 20 min at room temperature and then added to each well. The transfection mixture was removed from the wells after 7 h 30 min and the cells were washed with Opti-MEM (500 μl). The media was then replaced with 1 mL of antibiotic-free DMEM supplemented with 10% FBS. Five days post incubation, the media was replaced with fresh media (750 μL). After 24 h, the cells were stained with calcein AM (75 μL, 25 μM). Cells were incubated for 45 min and then washed with PBS (500 μL, 2 times). Microplate reading was performed to quantitate the number of attached cells. Excitation and emission wavelengths of 485 and 535 nm respectively were used. Results were plotted as bar graph (see figure S1).

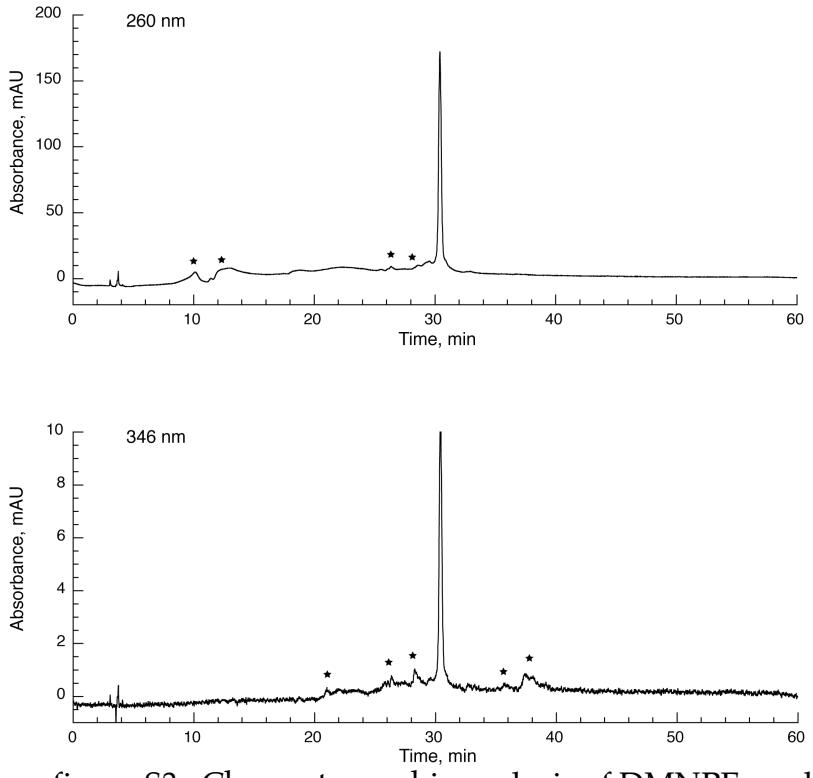


Supplementary figure S1: Bar graph indicating raw fluorescence signal of cells with varying concentration of pten and control siRNA.

HPLC chromatograms

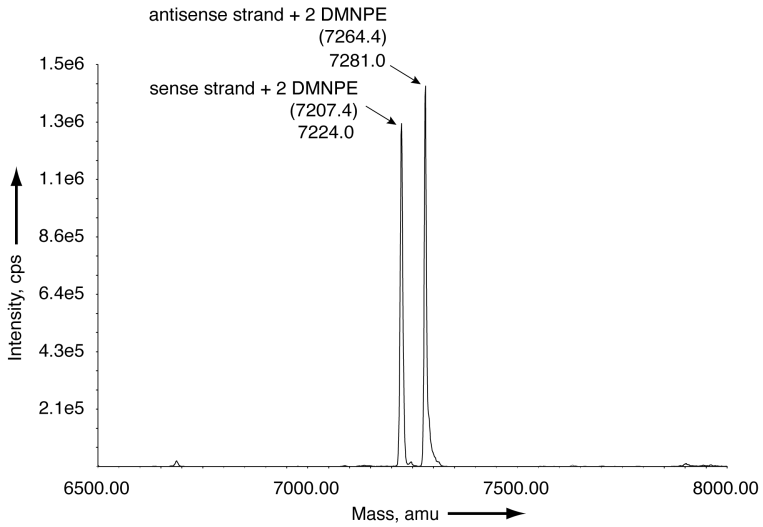


Supplementary figure S2: Chromatographic analysis of DMNPE modified pten-siRNA at 346nm. Starred peaks are also observed in the blank.



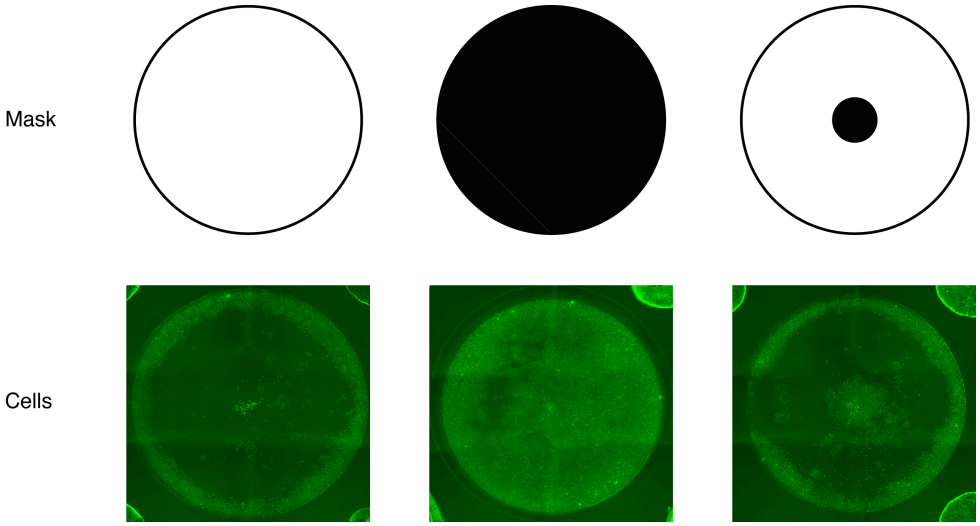
Supplementary figure S3: Chromatographic analysis of DMNPE modified control siRNA at 260nm and 346nm. Starred peaks are also observed in the blank.

ESI-MS spectrum



Supplementary figure S4: Deconvoluted mass spectrum of DMNPE tetra-modified control siRNA. Expected masses are show in parenthesis and the observed mass below.

Cell fluorescence images



Supplementary figure S5: Images showing replicates of pten transfected wells, irradiated using different masks as drawn above the images.

Reference

1. S. Shah and S. H. Friedman, *Nat. Protoc.*, 2008, 3, 351-356.