SUPPORTING INFORMATION:

NanoScript: A Nanoparticle-Based Artificial Transcription Factor for Effective Gene Regulation

Sahishnu Patel[1†], Dongju Jung[2†], Perry T Yin[3], Peter Carlton[2], Makoto Yamamoto[4], Toshikazu Bando[4], Hiroshi Sugiyama[2,4], and Ki-Bum Lee[1,3]*

[1] *Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA.*

[2] *Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kyoto, Japan.*

[3] *Department of Biomedical Engineering, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA*

[4] *Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, Japan*

[†] *Equal First Author Contribution*

*** Correspondence:**
Prof. Ki-Bum Lee
Department of Chemistry and Chemical Biology
Rutgers, The State University of New Jersey
Piscataway, NJ 08854 (USA)
Email: kblee@rutgers.edu
Website: http://rutchem.rutgers.edu/~kbleeweb/

MATERIALS AND METHODS

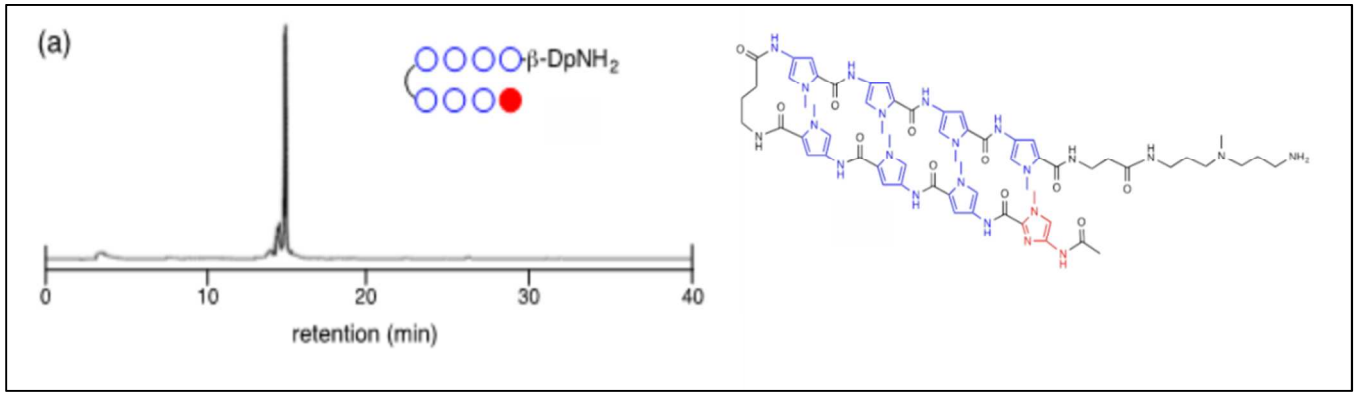
Synthesis of Synthetic Transcription Factors (STFs):

The transactivation peptide, having a sequence of CGSDALDDFDLDMLGSDALDDFDLDMLGS-NH_2, was purchased from Invitrogen. The nuclear localization peptide, having a sequence of CGGGPKKKRKVED-OH, was purchased from GenScript. All peptides were stored and dissolved as per the manufacturer recommendations.

All machine-assisted Py-Im polyamide synthesis was performed on a PSSM-8 peptide synthesizer (Shimadzu, Kyoto) with a computer-assisted operation system at 40 mg of Fmoc-β-Ala-Wang resin (ca. 0.55 mmol/g, 100~200 mesh, Novabiochem) by using Fmoc chemistry. Reaction steps in the synthetic cycle were as follows: i) deblocking steps for 4 min x 2, 20% piperidine in DMF; ii) coupling step for 60 min, corresponding carboxylic acids, 1H-Benzotriazolium, 1-[bis(dimethylamino)methylene]-5chloro-hexafluorophosphate (1-),3-oxide (HCTU) (88 mg), diisopropylethylamine (DIEA) (36 μL); iii) washing steps for 1 min x 5, DMF. Each corresponding carboxylic acids in coupling step were prepared in 1-methyl-2-pyrrolidone solution of Fmoc-Py-COOH (77 mg), Fmoc-Im-COOH (77 mg), Fmoc-PyIm-COOH (100 mg), Fmoc-γ-COOH (69 mg). All couplings were carried out with stirring by N2 gas bubbling. All lines were washed with DMF after solution transfers. After the completion of the synthesis by the last acetyl capping on the peptide synthesizer, the resin was washed with DMF and methanol, and dried in a desiccator at room temperature in vacuo. A dried resin was cleaved with 0.4 ml of 3,3'-diamino-N-methyldipropylamine for 3 h at 55 ºC. The reaction mixture was filtered, triturated from CH2Cl2 and Et2O, to yield crude as yellow powder. The crude was purified by flash column chromatography (elution with 0.1% trifluoroacetic acid in water and a 0-35% acetonitrile linear gradient (0-35 min) at a flow rate of 18 mL min-1 under 254 nm) to yield Py-Im polyamides as white powder.

(a) Analytical HPLC: Retention Time = 14.7 min (0.1 % TFA in water with CH3CN as eluent, and a linear gradient elution of 0-100% CH3CN over 40 min). 1H NMR (600 MHz, [D6]DMSO) δ 10.23 (s, 1H; NH), 9.97 (s, 1H; NH), 9.94 (s, 1H; NH), 9.93 (s, 1H; NH), 9.90-9.89 (t, J = 4.2 Hz, 3H; NH), 9.84 (s, 1H; NH), 8.10-8.04 (m, 3H; NH), 7.43 (s, 1H; CH), 7.28-7.21 (m, 3H; CH), 7.18-7.16 (m, 4H; CH), 7.07 (m, 3H;

CH), 6.91-6.87 (s, 4H; CH), 3.95 (s, 3H; NCH3), 3.86 (s, 3H; NCH3), 3.85 (s, 9H; NCH3), 3.84 (s, 3H; NCH3), 3.83 (s, 3H; NCH3), 3.81 (m, 3H; NCH3), 3.80 (m, 3H; NCH3), 2.74 (d, J = 4.2 Hz, 6H; CH2), 2.37-2.27 (m, 6H; CH2), 2.03 (s, 3H; COCH3), 1.91-1.90 (m, 4H; CH2), 1.81-1.76 (m, 6H; CH2). ESI-TOF-MS (positive) m/z calcd for C63H81N22O11+ [M+H]+ 1321.65; found 1321.64.



SPR Binding Affinity:

The Surface Plasmon Resonance (SPR) assays were performed using a BIACORE X instrument. Biotinylated hairpin DNAs were purchased from JBioS (Tokyo, Japan) and the sequences are shown in Tables. Hairpin biotinylated DNA are immobilized to streptavidin-coated sensor chip SA to obtain the desired immobilization level (approximately 900 RU rise). SPR assays were carried out using HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3mM EDTA, and 0.005 % Surfactant P20) with 0.1 % DMSO at 25 °C. A series of sample solutions with various concentrations were prepared in the buffer with 0.1 % DMSO and injected at a flow rate of 20 µl/min. To measure the rates of association (ka), dissociation (kd) and dissociation constant (KD), data processing was performed with an appropriate fitting model using the BIAevaluation 4.1 program. The 1:1 binding with mass transfer was used for fitting the sensorgrams to give better fitting. The closeness of fit is described by the statistical value: $\chi^2 = \frac{\sum_1^n (r_f - r_x)^2}{n-p}$ (rf = fitted value at a given point, rx = experimental value at the same point, n = number of data points, p = number of fitted parameters).

Cell Transmission Electron Microscopy (TEM):

HeLa cells were cultured with NanoScripts using the same method as above, but in a 6 cm cell culture dish. 48 hr post-transfection, the cells were trypsinized and fixed with Trump's Fixative (Electron Microscopy Sciences) for 1 hr, washed with sodium cacodylate buffer (Electron Microscopy Sciences), suspended in a 1% osmium tetroxide solution for 1 hr, washed with water, and then progressively dehydrated with ethanol (50, 70, 80, 95, 100%) . Then the cells were embedded in epoxy resin using the Low Viscosity Embedding Media Spurr's Kit (Electron Microscopy Sciences) following the manufacturer's protocol. The images were obtained with the JEOL 100CX TEM.

Reporter Plasmid Design:

The reporter plasmid was derived from the pSEAP2-Basic plasmid (Clontech), which lacks a eukaryotic promoter and enhancer sequences. Specifically, the STFM response element, which contains 6 sequences recognized by the DBD was cloned into the pSEAP2-Basic plasmid using the KpnI and HindIII restriction sites. The sequence of this STFM response element can be found in Figure S3. The sequence of the plasmid was confirmed by sequencing and restriction enzyme analysis.

PCR analysis:

Total RNA was extracted with TRIzol reagent (Invitrogen) and was reverse transcribed to cDNA with Superscript III Reverse Transcriptase (Invitrogen). Conventional quantitative RT-PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-time PCR System (Applied Biosystems). Primers sequences are included in the Supplemental Information (**Table S3**).

Immunocytochemistry:

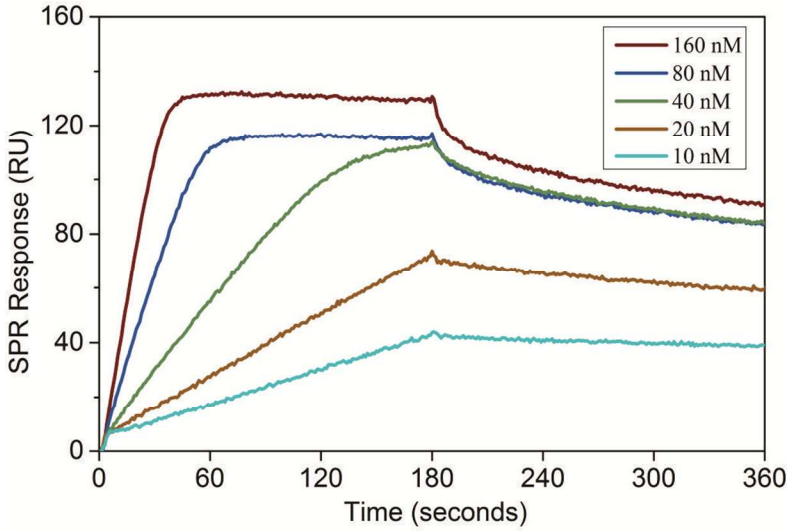
To investigate the nuclear localization of the dye-labeled (Alexa Flour 568, Life Technologies) NanoScript in HeLa cells 48 hr post-transfection, the media was removed and the cells were fixed for 15 minutes in formalin (Sigma) followed by two washes with PBS. The nucleus was stained with DAPI (Life Technologies) for 30 minutes and then washed with PBS three times. Slides were imaged on an OMX microscope in widefield (non-SIM) mode. A 405nm / 200mW laser was used to excite DNA stained with DAPI, and a 593nm / 500mW laser was used to excite the Texas Red dye. 200msec exposures were used. Z stacks were taken from 8 to 10μm depending on cell Z depth, at 0.125μm section thickness. Emission light was collected with a 100x, 1.4NA oil immersion objective (Olympus) and emission channels were directed onto one of two separate Photometrics CascadeII:512 EMCCD cameras, with a final lateral pixel size of 0.0792μm. Constrained iterative deconvolution was used with a measured point spread function to de-blur images. Images from separate cameras were aligned in XY using a transformation matrix derived from previously recorded images of 100nm fluorescent latex beads, and in Z using the DAPI image recorded onto both cameras.

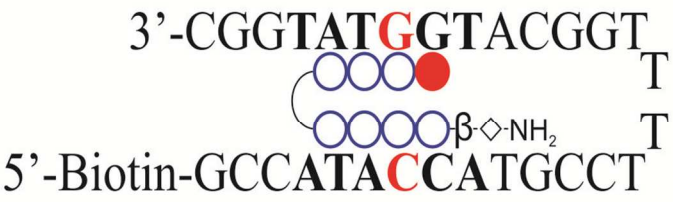
Sample Preparation and ICP-OES Measurement:

After HeLa were seeded in a 24-well plate (20,000 cells/well), 1 nM of NanoScript was added; and after 4 hours, the cells were washed with PBS three times and detached using trypsin. After centrifugation, the supernatant was removed, and the cells were resuspended in PBS and counted using a hemocytometer. After centrifugation, the supernatant was removed and 25 μL of lysis buffer was added to the cell pellet. Then 250 μL of aqua regia (caution: extremely reactive) was added and allowed to sit overnight in an uncapped eppendorf tube. The next day, the solution was transferred to 9.725 mL distilled water (10 mL total volume with 2.5 % aqua regia). Cellular uptake experiments were performed three times and each replicate was measured for gold content three times by Inductively coupled plasma optical emission spectroscopy (Perkin Elmer Optima 7300 DV), operating under established conditions. A standard curve comparing the number of gold nanoparticles to the Au intensity was established using known nanoparticle concentrations.

SUPPORTING FIGURES AND TABLES

FIGURE S1: BINDING AFFINITY OF POLYAMIDE TO MISMATCH DNA SEQUENCE: SPR sensorgrams show the interaction of varying polyamide DBD concentrations with complementary hairpin DNAs. The equilibrium constant (K_D), which is indicative of the binding affinity, was determined by the ratio of the dissociation constant (k_d) to the association constant (k_a). The mismatched G-C base pair is highlighted in red. Comparing the binding affinity of the polyamide to the target sequence (from Figure 2) versus the mismatch sequence, there is a 70 fold difference.





k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
1.54×10^4	1.76×10^{-3}	**1.14×10^{-7}**

Affinity of Polyamide to Target Sequence = **1.6×10^{-9} M**
Affinity of Polyamide to Mismatch Sequence = **1.14×10^{-7} M**
Difference of Over **70 Fold**

FIGURE S2: UV ABSORBANCE OF NANOSCRIPT CONJUGATES. Unmodified, citrate-stabilized 10nm AuNPs shows a characteristic peak at 518 nm. After functionalization with MUA and conjugation of STF components, the successive shift in the absorbance peak implies attachment of biomolecules on the AuNP surface.

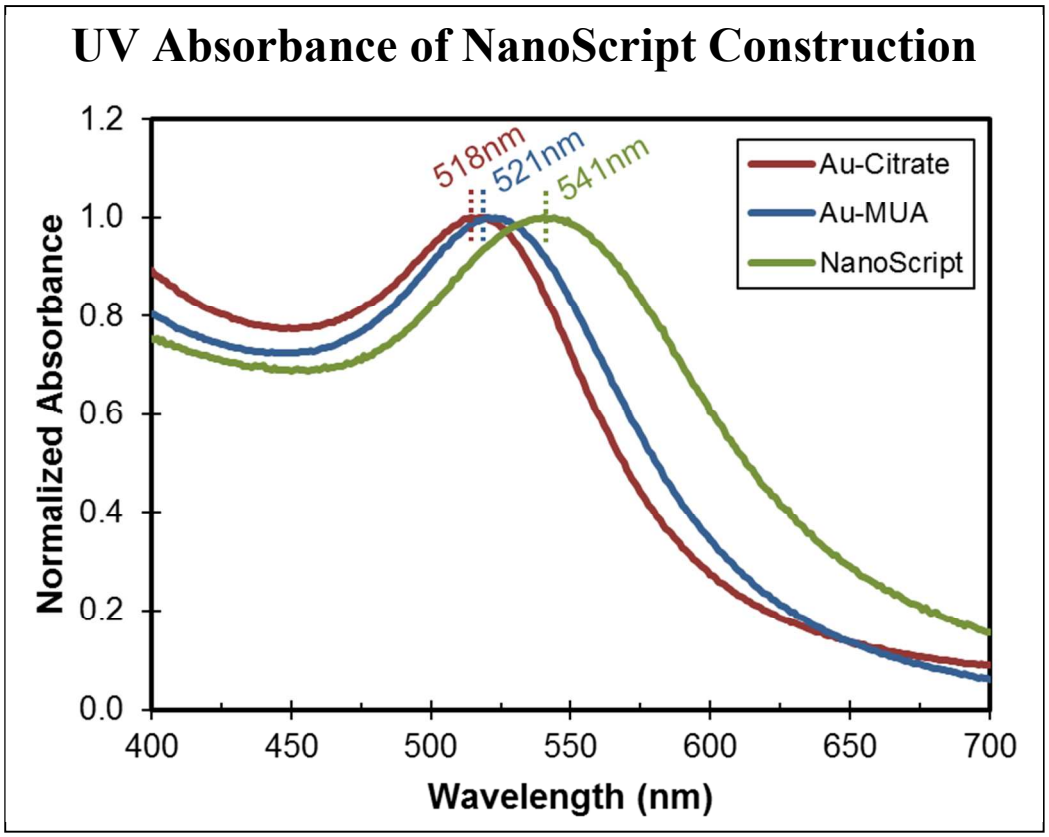


TABLE S1: THE LIGAND RATIO ON NANOSCRIPT. HPLC analysis was performed to calculate the moles of each peptide in both the solution before conjugation and the supernatant solution after conjugation containing unreacted peptides. The mole ratio of the difference is the ratio on the peptide on NanoScript.

	Mole Ratio of STFs on NanoScript Surface
NLS	68.2 ± 1.0%
TAP	22.8 ± 2.6%
DBD	9.0 ± 2.1%

FIGURE S3: NANOSCRIPT STABILITY. The MUA-modified nanoparticles and NanoScript were maintained at 37°C in various solutions including: water pH=5.5, PBS pH=7.4, and Cell Culture Media. Over the course of three days, the stability of the nanoparticles was tested using UV-Vis, which revealed only a slight shift in the peak and indicated that the nanoparticles remained stable in these solutions.

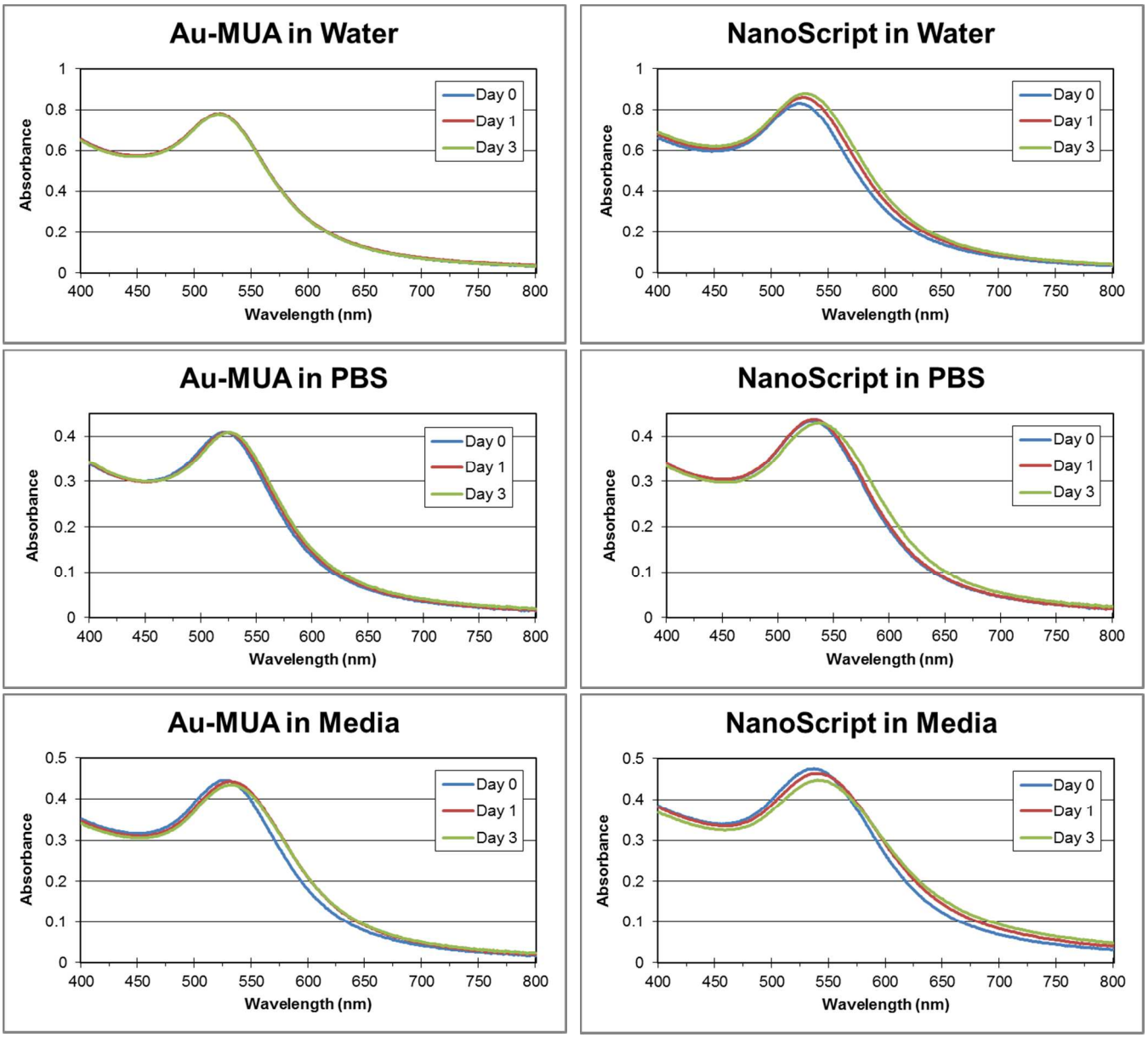


FIGURE S4: NUCLEAR LOCALIZATION OF NANOSCRIPT. HeLa cells were transfected with NanoScript and imaged 48 hr later. The fluorescence image shows the overlap of NanoScript (red) with the nucleus (blue). (Scale bar = 20 μm)

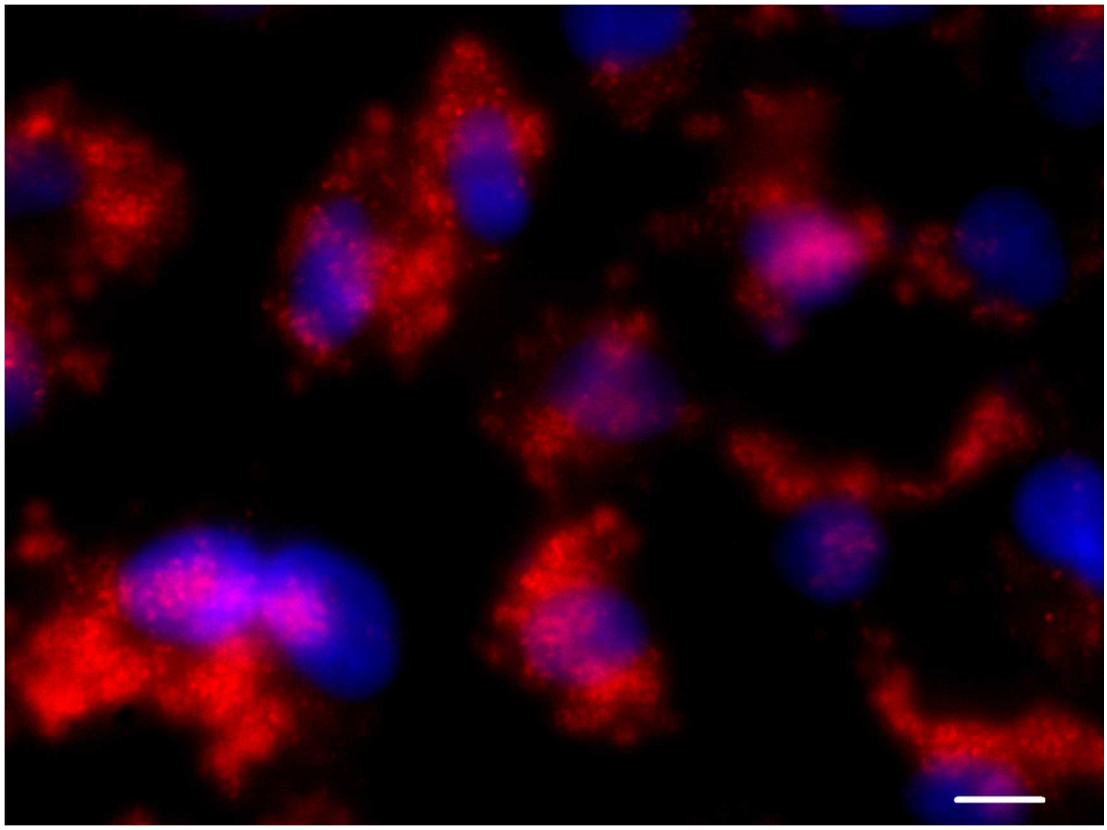


FIGURE S5: PHASE CONTRAST IMAGE OF HELA CELLS. A phase contrast image of HeLa cells, taken 48 hr post-transfection, confirms that the HeLa cell morphology seems to remain intact without any damage to the plasma membrane.

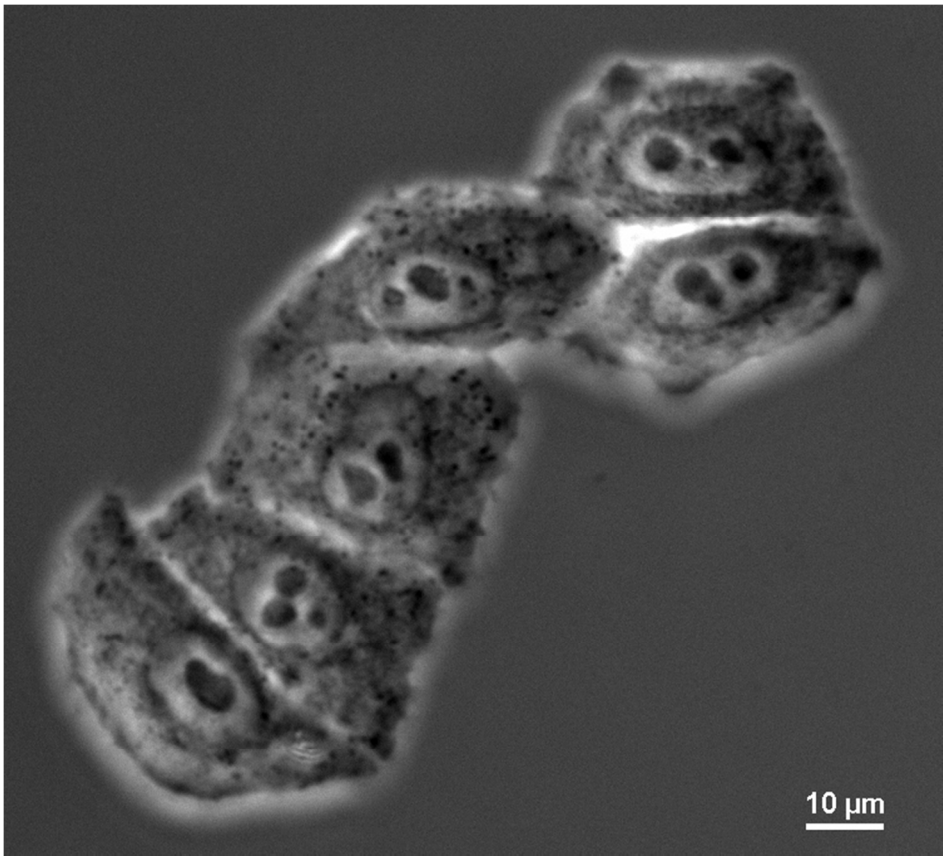


FIGURE S6: CELL TEM OF WHOLE CELL. A cross-sectional TEM micrograph showing a HeLa 48 hr post-transfection reveals that NanoScript (indicated with light blue arrows) is localized in the cytoplasm, near the outside of the nuclear membrane, and within the nucleus (purple outline).

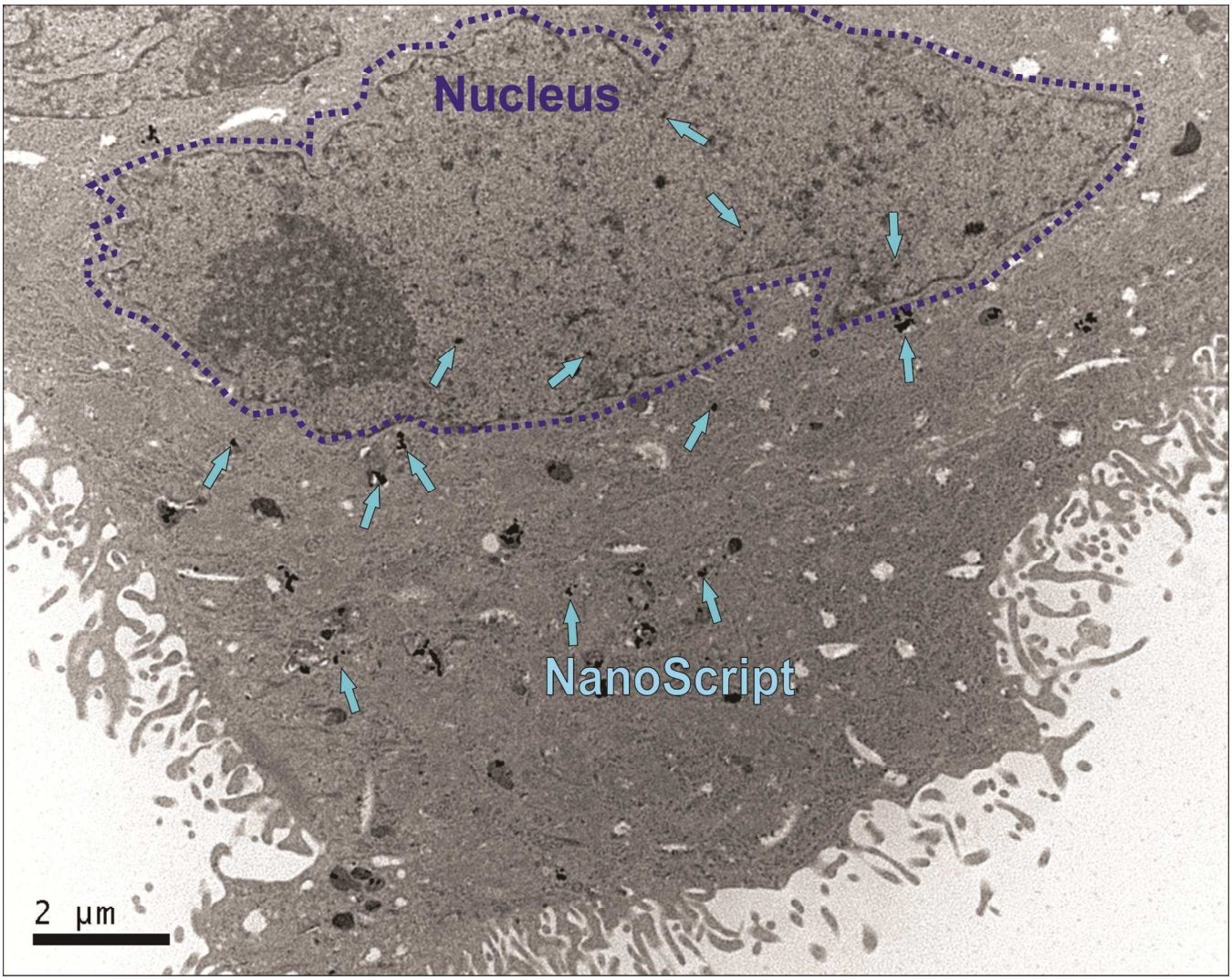


FIGURE S7: REPORTER PLASMID RESPONSE ELEMENT. Response element containing six copies of the targeted sequence (TGTTAT) that secrets alkaline phosphatase upon transcription is located 36 base pairs upstream from the TATA box. This response element was inserted in the pSEAP2-Basic Vector (Clontech), between KpnI (GGTACC) and HindIII (AAGCTT) sites.

5′-AGC**GGTACC**GCTAGCAGCTT**ATAACA**TTCCATA**TGTTAT**AC**ATAACA**TTC

CATA**TGTTAT**AC**ATAACA**TTCCATA**TGTTAT**ACGTCGACAAGCTATGAGATCT

AGACTCTAGAGGG**TATA**TAATGGAAGCTCGACTCCAGCTTGGCATTCCGGT

ACTGTTGGTAA**AAGCTT**CGAATA-**3′**

FIGURE S8: CELLULAR VIABILITY OF NANOSCRIPT TREATED CELLS. Cellular viability was calculated by analyzing MTS assay activity 48 hr post-transfection. The graph shows the cell viability of various NanoScript concentrations as well as NanoScript controls that lack individual components (w/o = without). Percentages are compared to untreated cells and standard error is calculated from three independent trials.

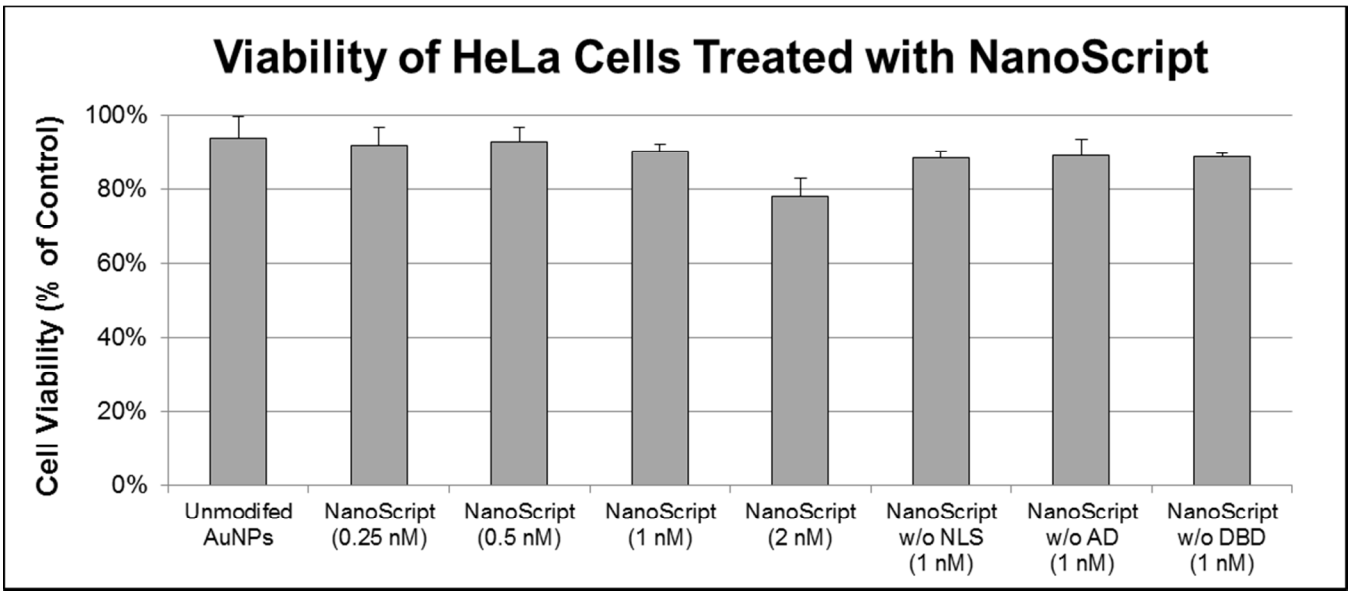


TABLE S2: CONSENSUS PROMOTER SEQUENCES ON NATIVE DNA. Number of binding sites that the polyamide DBD can bind on the promoter sequence of the four overexpressed endogenous genes. Binding sites are within 1000 basepairs 5' from the transcription start site.[17]

Gene	Number of Binding Sites on Promoter Region
LGALS8	36
PPAP2A	22
BAG4	11
EGLN3	28

TABLE S3: PRIMER SEQUENCES AND THE EXPECTED BAND SIZE.

Target	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Expected Size (bp)
LGALS8	CCTATGACACGCCTTTCAAAAGA	CAGCACCATAATCACGATCTCAA	56
PPAP2A	GGCAGGTTGTCCTTCTATTCAG	CAGTGTGGGGCGTAAGAGT	120
BAG4	ACTTACCGTTCATCTGGCAAC	GGGTGCTTCAGTCTGACAGT	81
EGLN3	TCCTGCGGATATTTCCAGAGG	GGTTCCTACGATCTGACCAGAA	93

VIDEO S1: NUCLEAR LOCALIZATION 3D VIDEO

(Please refer to video)