# Electronic Supplementary Information

## DNAzyme activated protein-scaffolded CRISPR-Cas9 nanoassembly for genome editing

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**Experimental Section:**

**Chemicals and materials**

DNA marker, Spin Column RNA Cleanup & Concentration Kit, MTT Cell Proliferation and Cytotoxicity Assay Kit, plasmids pUC57-GFP with and without restriction enzyme cutting site of BamHI encoding EGFP from Sangon Biotech Co., Ltd. (Shanghai, China). DNA oligonucleotides in this work were purchased from TaKaRa Biotech. Inc. (Dalian, China). The sequences of these plasmids and DNA are given in Table S1. EnGenTM Cas9 NLS, S. pyogenes, BamHI and streptavidin were obtained from New England Biolabs LTD. (Beijing, China). AmpliScribe™ T7 High Yield Transcription Kit was acquired from Epicenter (Madison, U.S.A.). The GeneJET Gel Extraction Kit was obtained from Thermal Scientific (Shanghai, China). All other chemicals used in the work were of analytical grade and used as received without any further purification. The solutions were prepared with ultrapure water obtained from a Milli-Q water purification system (resistance > 18.25 MΩ) (Billerica, MA).

## Instruments

The fluorescence measurements were visualized at room temperature via an FL-7000 spectrometer (Hitachi, Japan). Real-time fluorescence intensity obtained from C1000 Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.) with a CFX96. Agarose gel was performed on a Tanon 4200SF gel imaging system (Tanon Science & Technology Co. Ltd., China). All confocal images were acquired using an oil dipping objective (60×) on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). Flow cytometric analysis of cells was carried out on a CytoFLEX™ flow cytometer (Beckman Counter, Inc., U.S.A.).

## Transcription and Purification of Single-guide RNA (sgRNA)

The sgRNA was transcribed with AmpliScribe™ T7 High Yield Transcription Kit in vitro according to the manufacturer's instructions. The transcription template encoding a T7 promoter and sgRNA were built into the pUC57-GFP plasmid, and the plasmids was linearized with BamHI, purified using GeneJET Gel Extraction Kit, in which the sgRNA containing a 20 bp EGFP or CD 71 targeting sequence (Table S1). The transcribed RNA was extracted and purified by Spin Column RNA Cleanup & Concentration Kit, and then the purified RNA was quantified with Nanodrop 2000c (Thermo Scientific) and analyzed by agarose gel electrophoresis.

## Plasmid Cleavage Assay to Detect the Activity of Cas9

Plasmids pUC57-GFP encoding EGFP target sequence was used as the substrate for Cas9 activity assay. A 30 μL reaction in 1 × Cas9 nuclease reaction buffer containing 300 ng circular plasmid, 100 nM purified Cas9 and 100 nM sgRNA. After incubation for 1.5 h at 37 °C, the plasmid was analyzed by 0.8% agarose gel electrophoresis.

## Assembly and Characterization of CRISPR-Cas9 Nanoassembly

The CRISPR nanosystem is assembled from a Y-shaped DNA (Y-DNA) and a DNAzyme motor. The 3' end of the Yc strand is functionalized with a biotin molecule for immobilization onto the SA, and the rational engineered Ya and Yb were hybridized with the 3' sticky end of the substrate of deformed 8-17E DNAzyme whose 5' end ssDNA motifs were partially hybridized with sgRNA. Afterward, all the strands were (the molar ratio of Ya, Yb and Yc were at 1:1:1; the molar ratio of DNAzyme catalytic strand and its substrate strand were at 1:1) hybridized by heating and annealing with 10 mM Tris-HCl buffer (pH 7.4, 150 mM NaCl) followed by incubation at 37 °C with SA and Cas9, and the molar ratio of SA, Y-DNA, DNAzyme, sgRNA and Cas9 were at 1:4:8:8:8. The CRISPR nanoassembly were further diluted to give a concentration of sgRNA at 100 nM in RNAase free water for

particle characterization or culture medium for cell study. Size and zeta potential of CRISPR nanoassembly were measured by Malvern Zetasizer Nano ZS90 Zetasizer (Nano ZS, Malvern). For negative-stain electron microscopy micrographs, the CRISPR nanoassembly were dropped onto a TEM copper grid and stained with 2% uranyl acetate. TEM images were acquired from JEM-2100F with an accelerating voltage of 200 kV.

**Determination of Cell Viability**

Cell viability of HeLa-eGFP cells delivered with Cas9-bounded sgRNA were analyzed using an MTT Cell Proliferation and Cytotoxicity Assay Kit. The cells (~25000 cells/well) were seeded in 96-well plates and incubated at 37 °C in 5% $CO_2$ atmosphere for 24 h. Subsequently, the cells were incubated for another 24 h with a given dosage of CRISPR nanoassembly with different concentrations of Cas9, then 10 μL MTT (5 mg•mL$^{-1}$) was added to each well and incubated for 4 h. After removing the cell culture medium, 100 μL Formazan solution was added to each well for dissolving crystals formed in living cells. Another 4 h later, the absorbance at 570 nm was measured on a Thermo Scientific Multiskan Microplate Reader (Thermo Fisher, U.S.A.) to calculate the cell viability.

**Cell Culture and Fluorescence Imaging**

HepG2 cells, MDA-MB-231 cells and HeLa-eGFP cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal bovine serum (FBS), 100 μg•mL$^{-1}$ penicillin and 100 μg•mL$^{-1}$ streptomycin at 37 °C in a humidified incubator containing 5 wt %/vol $CO_2$. HeLa cells were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% FBS, 100 μg•mL$^{-1}$ penicillin and 100 μg•mL$^{-1}$ streptomycin. The cell number was determined using a hemocytometer. HepG2 cells, MDA-MB-231 cells and HeLa cells were seeded in a 35 mm confocal dish and cultivated in 2 mL of fresh full serum medium containing 10% fetal bovine serum for 24 h at 37 °C. For estimating the operation of the DNAzyme in the CRISPR nanoassembly within Cells, the nanoassembly (100 μL) were added to 900 μL Opti-MEM medium (Life Technologies) and incubated with the cells for 3 h at 37 °C. After that, the cells were washed with 1 × PBS buffer for three times. Finally, 0.1 mg•mL$^{-1}$ Hoechst and 1 × PBS buffer were added then incubated with the cells for 5 min at 37 °C. The cells were then subjected to confocal microscopic observation. For time-correlated experiments, the cells were stained with Hoechast first, followed by washing three times with 1 × PBS buffer. Then, the nanoassembly (100 μL) were added to 900 μL Opti-MEM medium and incubated with the cells for real time fluorescence imaging using a confocal laser scanning fluorescence microscope.

**Gene Editing in HeLa-eGFP Cells**

To evaluate intracellular gene editing capability of the CRISPR nanoassembly, HeLa-eGFP cells were seeded into 35 mm confocal dish and cultivated in 2 mL of fresh full serum medium for 24 h at 37 °C. Then the CRISPR nanoassembly (100 μL) were added to 900 μL Opti-MEM medium and incubated with the cells for 3 h at 37 °C. After that, the cells were washed with 1 × PBS buffer for three times and the medium was replaced with fresh full serum medium. After the delivery for two days, the cells were stained with Hoechst and then analyzed using confocal microscopic observation and flow cytometric analysis.

**DNA Sequencing to Detect Genomic Mutations**

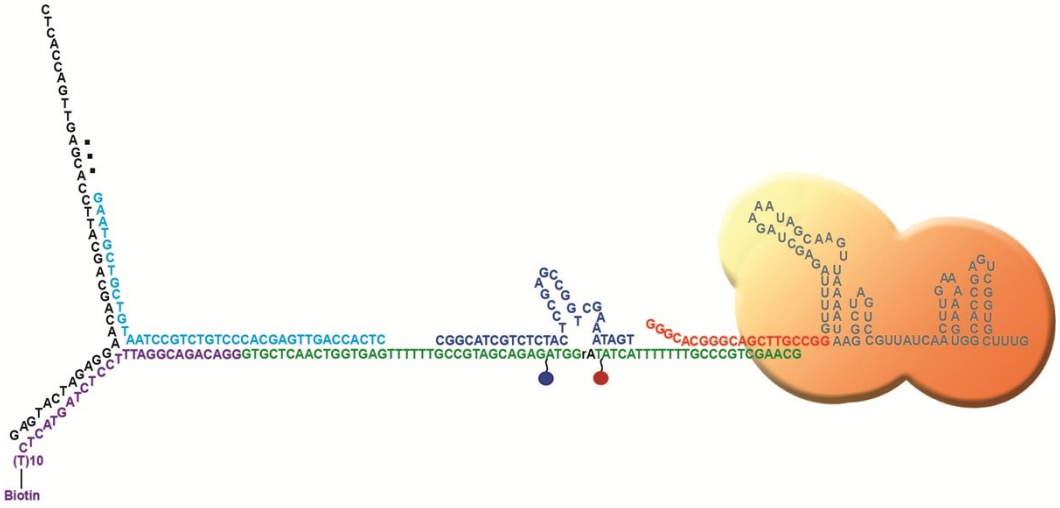
Purified PCR amplicons of the T7EI assay were cloned into Zero Blunt TOPO DNA sequencing vectors (Life Technologies). The cloned plasmids were purified by GeneJET Plasmid Miniprep Kit and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China) with T7 promoter primer.

**Table S1.** Oligonucleotide sequences of nucleic acid probes used in the study.

| **Oligonucleotides** | **Sequences (5'→3')** |
|---|---|
| Ya | GAGTACTAGAGGAACAGCAGCATTCCACGAGTTGACCA CTC |
| Yb | GAATGCTGCTGTAATCCGTCTGTCCCACGAGTTGACCAC TC |
| Yc linked to SA | GGACAGACGGATTTCCTCTAGTACTCTTTTTTTTTT-biotin |
| DNAzyme strand | CGGCATCGTCTCTACTCCGAGCCGGTCGAAATAGT |
| Mutant DNAzyme strand | CGGCATCGTCTCTACTCCGATCCGGTCTAAATAGT |
| Substrate strand (eGFP) | GCAAGCTGCCCGTTTTTTTACT(Cy5)ATrAGGT(BHQ3)AG AGACGATGCCGTTTTTTGAGTGGTCAACTCGTG |
| Substrate strand (CD71) | CACACTAGCGCGTTTTTTTACT(Cy5)ATrAGGT(BHQ3)AG AGACGATGCCGTTTTTTGAGTGGTCAACTCGTG |
| eGFP RNA transcription template | **TAATACGACTCACTATAGG**GCACGGGCAGCTTGCCGG GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC TTTGGATCC<br>**T7 Promoter** eGFP Targeting, restriction enzyme cutting site of BamHI |
| CD71 RNA transcription template | **TAATACGACTCACTATAGG**GACGCGCTAGTGTGAGTG CGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTA GTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG CTTTGGATCC<br>**T7 Promoter** CD71 Targeting, restriction enzyme cutting site of BamHI |

**Scheme S1.** a) The formation of Y-shaped DNA, and the loading of DNAzyme and sgRNA/Cas9. b) Illustration of the operation processes of the DNAzyme.

a)




b)

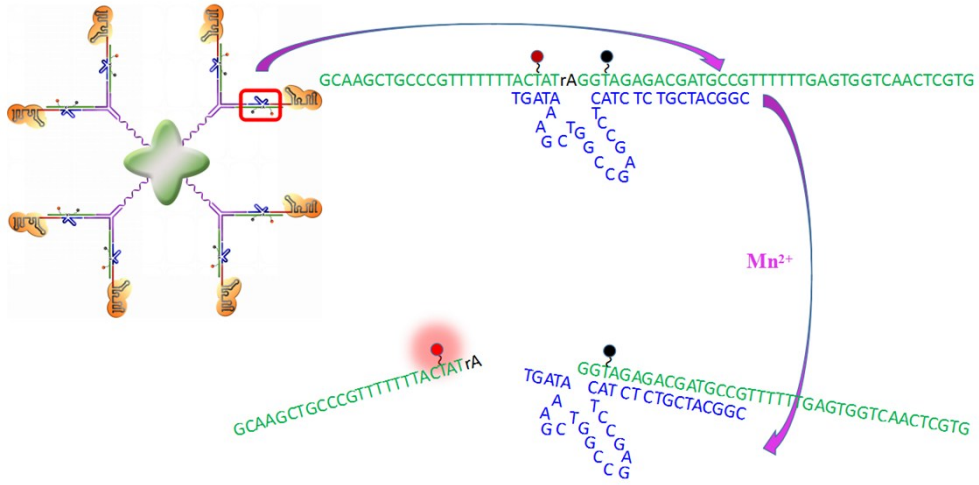


**Figure S1.** Gel electrophoresis images of plasmid and sgRNA. a) Line 1: the circular plasmid that containing a restriction site of BamHI, line 2: the circular plasmid in line 1 was incubated with BamHI for 2 h at 37 °C. b) Agarose gel electrophoresis analysis of T7 RNA polymerase product, purified sgRNA of eGFP.

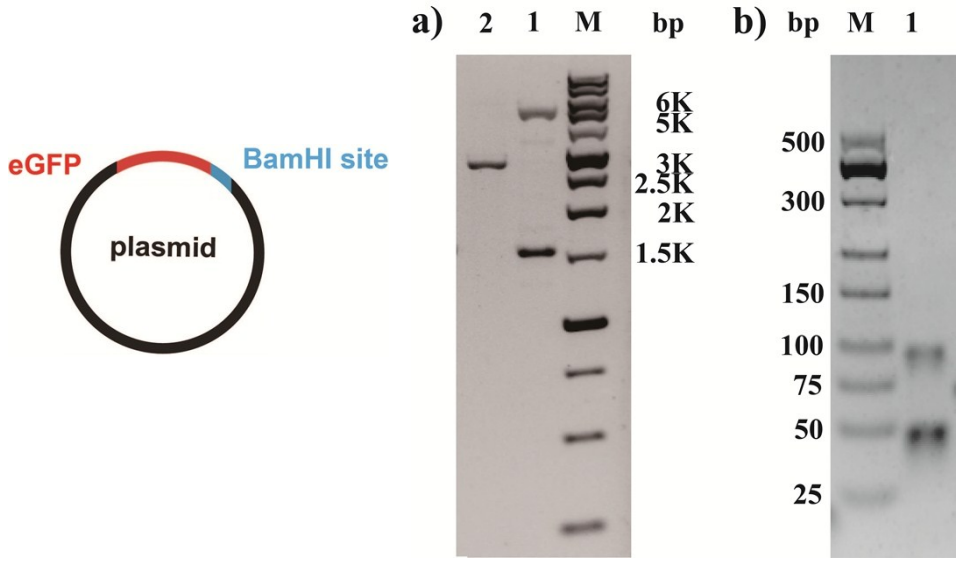


**Figure S2.** Hydrodynamic size ditribution of the nanoassembly.

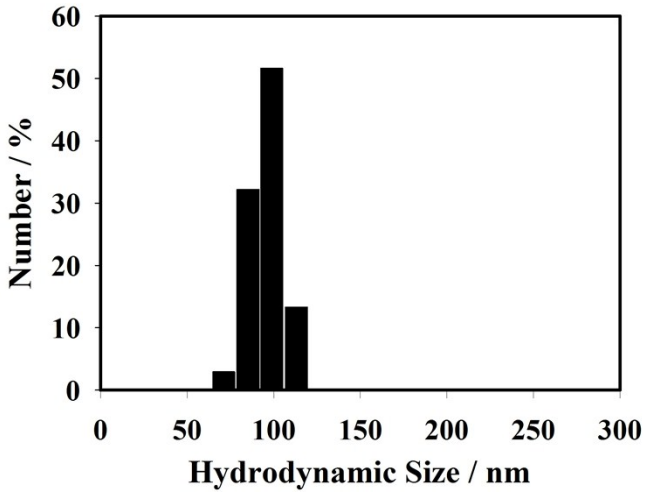


**Figure S3.** a) Fluorescence emission spectra of the nanoassembly with the addition of different concentrations of $Mn^{2+}$ ranging from 0 to 2 mM. b) Fluorescence intensity for the nanoassembly with respect to the $Mn^{2+}$ concentrations.

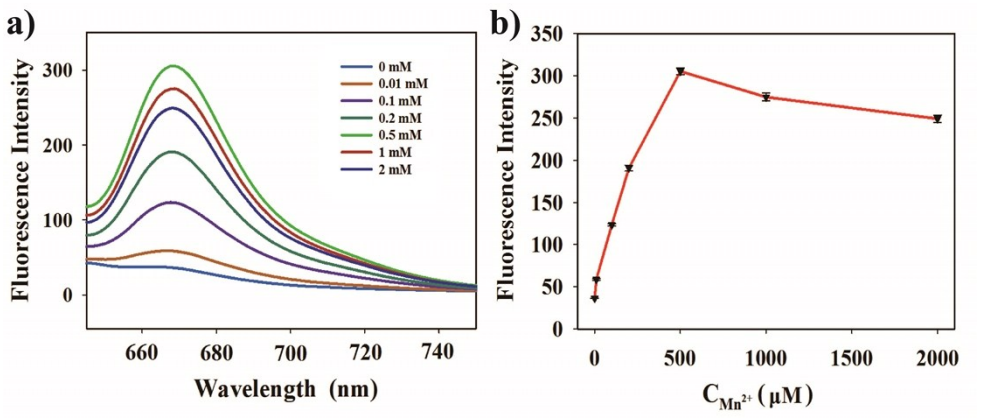


**Figure S4.** Response of the DNAzyme in the nanoassembly to different divalent metal ions. The concentrations were 0.5 mM $Mn^{2+}$, 5 mM $Ca^{2+}$ and $Mg^{2+}$, 0.2 mM $Pb^{2+}$ and 0.01 mM $Zn^{2+}$.

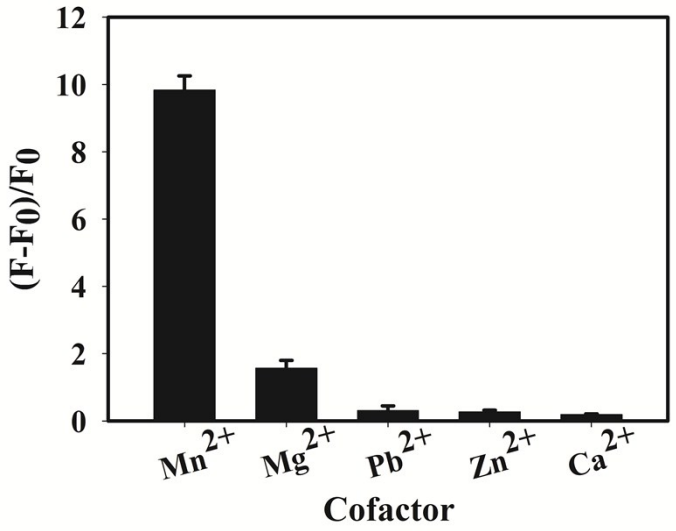


**Figure S5.** Cell viability assay of HeLa-eGFP cells treated with CRISPR-Cas9 nanoassembly.

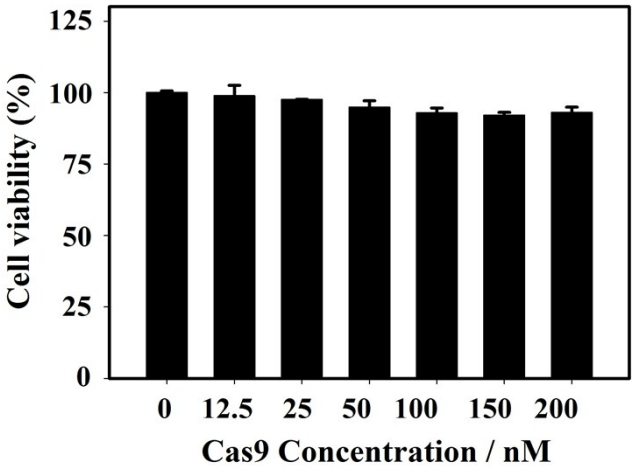


**Figure S6.** Time-dependent CLSM images of HeLa cells incubated with Cy5-labeled CRISPR-Cas9 nanoassembly.

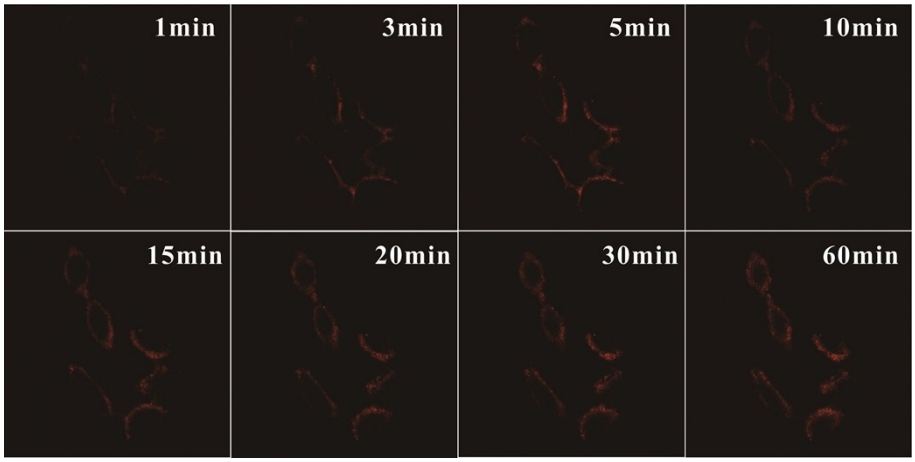


**Figure S7.** The investigation of intracellular endosomal escape of CRISPRCas9 nanoassembly. a) Fluorescence images of HeLa cells after incubation with Cy5-labeled nanoassembly (red) for 0, 30 and 60 min. Lysotracker was used to stain the endosomes. b) Flow cytometric assay of HeLa with varying incubation time. c) Intracellular localization assay of Cy5-labeled nanoassembly with Lysotracker in HeLa cells after incubation with nanoassembly for 60 min. d) Fluorescence intensity profile of regions of interest (white line in c).

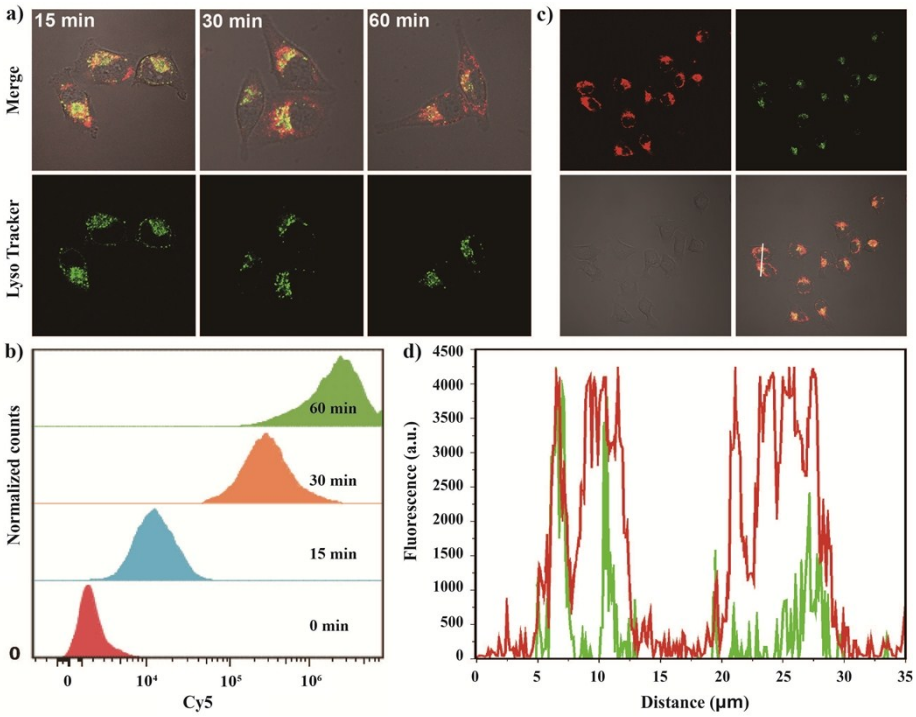


**Figure S8.** Z-stack images of HeLa cells incubated with Cy5-labeled CRISPR-Cas9 nanoassembly (red) for 60 min followed by Lysotracker (green) staining.

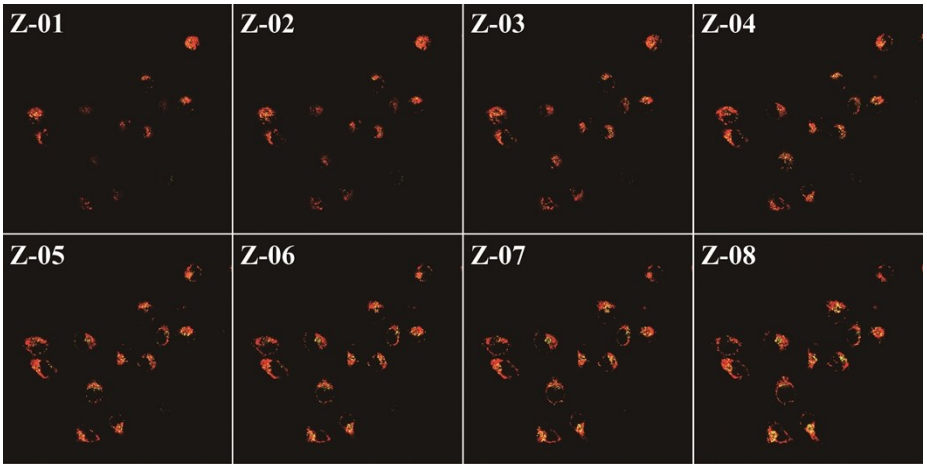


**Figure S9.** Time-dependent CLSM images of MDA-MB-231 cells incubated with $Mn^{2+}$ and CRISPR-Cas9 nanoassembly successively. Red: Cy5; blue: nucleus stained with Hoechst.

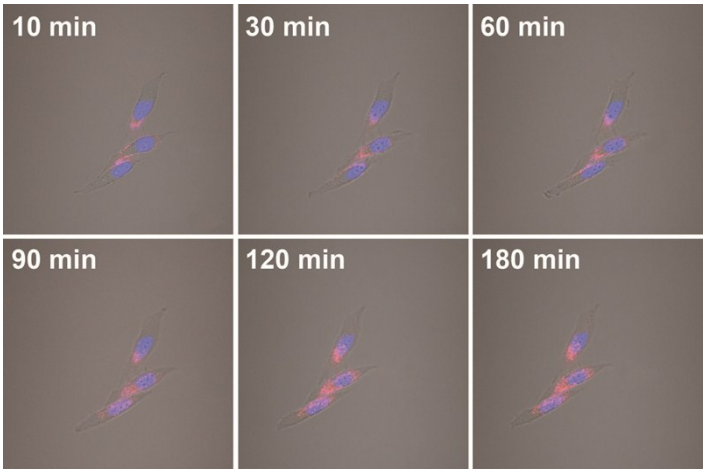


**Figure S10.** Z-stack images of HeLa cells after incubation with $Mn^{2+}$ for 1 h and CRISPR-Cas9 nanoassembly for 3 h at 37 °C successively. Red: Cy5; blue: nucleus stained with Hoechst.

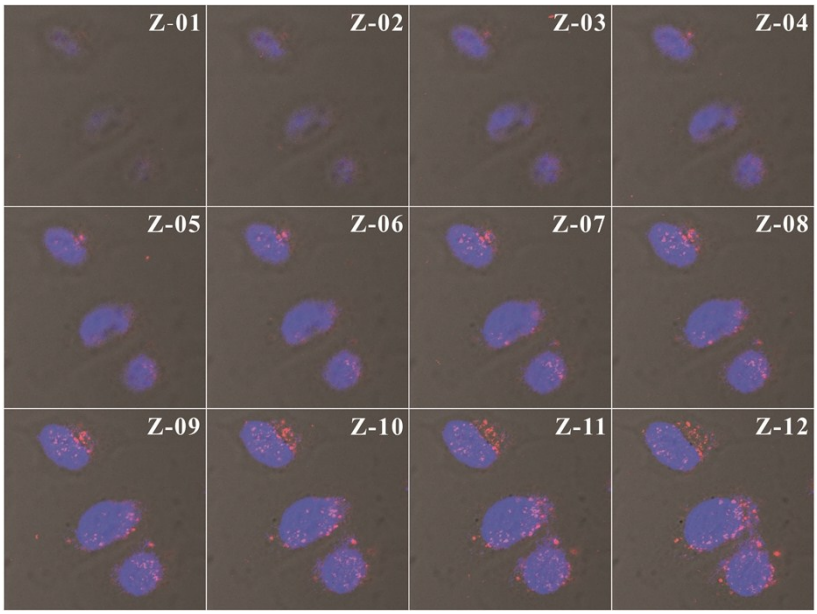


**Figure S11.** (a)Time-dependent CLSM images of HeLa cells only incubated with CRISPR-Cas9 nanoassembly. Blue: nucleus stained with Hoechst. (b) Flow cytometric assay of HeLa cells with CRISPR-Cas9 nanoassembly in the absence (red) or presence (blue) of 500 μM $Mn^{2+}$.

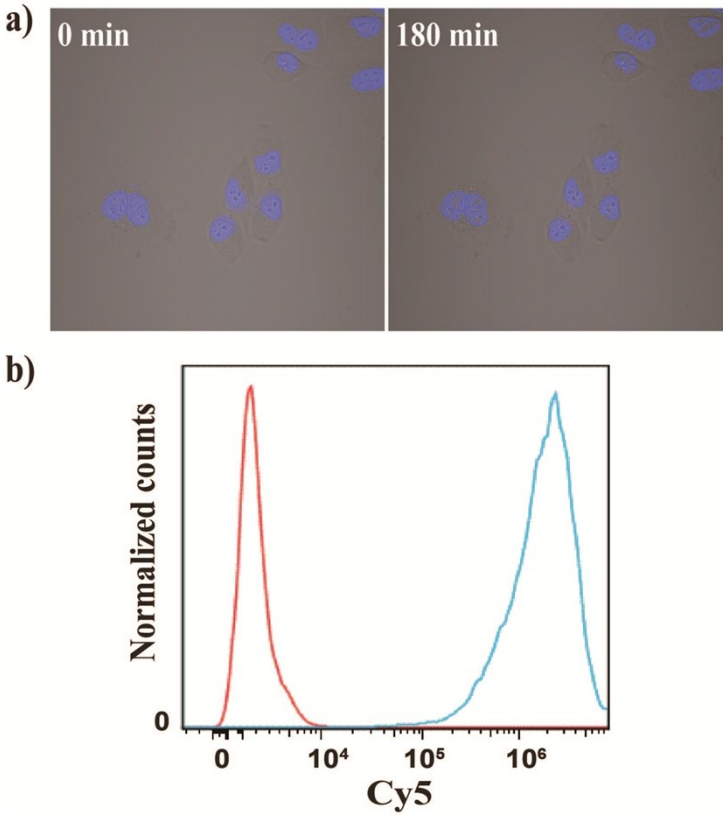


**Figure S12.** Imaging of HeLa cells after uptake of the CRISPR-Cas9 nanoassembly. a) Images showing HeLa cells after incubation with $Mn^{2+}$ and CRISPR-Cas9 nanoassembly that just contained substrate strand but not DNAzyme catalytic strand (negative control). b) Images showing HeLa cells after incubation with $Mn^{2+}$ and CRISPR-Cas9 nanoassembly that contained a mutant DNAzyme catalytic strand (negative control). c) Images showing HeLa cells after incubation with only CRISPR-Cas9 nanoassembly (negative control). d) Images showing HeLa cells after incubation with $Mn^{2+}$ and CRISPR-Cas9 nanoassembly.

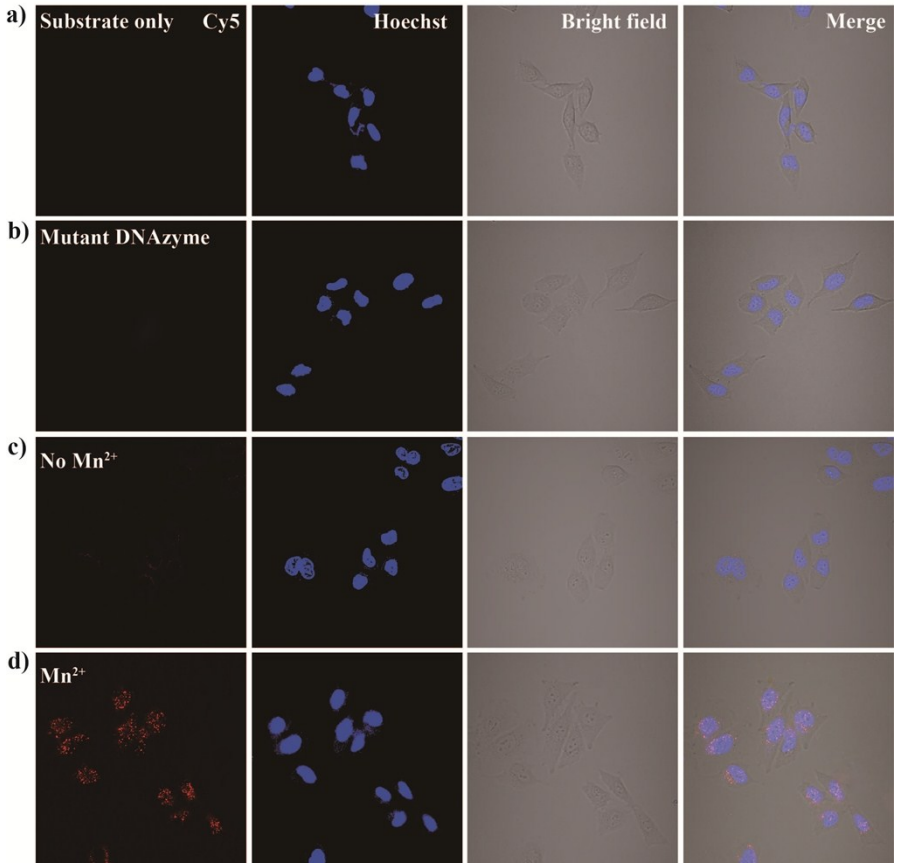


**Figure S13.** DNA sequencing of Cas9/sgRNA targeted gemonic locus in HeLa-eGFP cells. Target site and PAM site are shown in blue and purple, respectively. For each example shown, the unmodified genomic site is the first sequence, and the number of insertion/deletion as compared to the wild type sequence is shown on the left.

Wild type GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

Deletions

-16 GATGCCACCTACGGCAAGCTGACCCTGAAGTTC----------AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

-11 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG----------TGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

-10 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCG----------CGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

-9 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCC----------CCTCGTGACCACCCTGACCTACGGC

-4 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGC----------TGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

-2 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGC------CACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

-2 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC------CGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

-2 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGG------AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

Insertions

+2 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCCAGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

+7 GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGTTCATCTGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

**Figure S14.** DNA sequencing of Cas9/sgRNA targeted gemonic locus in HeLa-CD71 cells. Target site and PAM site are shown in blue and purple, respectively. For each example shown, the unmodified genomic site is the first sequence, and the number of insertion/deletion as compared to the wild type sequence is shown on the left.

Wild type AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGACGCGCTAGTGTGAGTGCGGGCTTCTAGAACTACACCGACCCTCGTGTCCTCCCTTCATCC

Deletions

-17 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGACGCGCTAGTGTGAG----------ACACCGACCCTCGTGTCCTCCCTTCATCC

-14 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGACGCGC----------GCTTCTAGAACTACACCGACCCTCGTGTCCTCCCTTCATCC

-11 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGG----------TGTGAGTGCGGGCTTCTAGAACTACACCGACCCTCGTGTCCTCCCTTCATCC

-7 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGACGCGCTAGTGTGAGTGCGGGC----------ACTACACCGACCCTCGTGTCCTCCCTTCATCC

-2 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGA-----CGCTAGTGTGAGTGCGGGCTTCTAGAACTACACCGACCCTCGTGTCCTCCCTTCATCC

Insertions

+3 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGACGCGCTAGTGTGAGTGCGGATGGCTTCTAGAACTACACCGACCCTCGTGTCCTCCCTTCATCC

+3 AGAGCGTCGGGATATCGGGTGGCGGCTCGGGACGGAGGACGCGCTAGTGTGATCAGTGCGGGCTTCTAGAACTACACCGACCCTCGTGTCCTCCCTTCATCC