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Cytosolic delivery of CRISPR/Cas9 ribonucleoproteins for genome editing using chitosan-coated red fluorescent protein nanoparticles

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Experimental

Materials. Chitosan oligosaccharide (COS, deacetylation degree of 90% and MW = 1 kDa) was purchased from Golden-Shell Biochemical Co., Ltd (Zhejiang, China). Glutaraldehyde was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). FITC was purchased from Solorbio (Shanghai, China). The Lipofectamine CRISPMAX was purchase from Thermo Fisher (China). The serum mediums were purchase from Natocor (Wuhan, China). All the other chemical reagents used were purchased from Sigma-Aldrich (Mainland, China). The *E. coli* Rosetta (DE3) strain, the HEK293, HEK293T, HeLa, RAW264.7, U2SO and A549 cell lines were purchased from BioVector NTCC Inc (China).

Construction of Cas9 RNP and engineered Cas9 RNP expression plasmids. The expression plasmid of original Cas9 RNP was constructed based on pCold Ⅰ (Takara) plasmid by replacing the original cspA promoter with a tac promoter, and by replacing the f1 ori with a T7 promoter. The spCas9 gene from pET-Cas9-NLS-6xHis (addgene plasmids#62933) and the sgRNA sequences (~100 bp) were inserted successively, forming the plasmid Ptac-Cas9-T7-sgRNA. The vector contains a gRNA transcription element that can be easily replaced with new gRNA by SalI digestion. Then, we added twenty glutamine residues in the N terminus of the above plasmid using overlap-extension PCR, obtaining the plasmid termed E-Cas9 RNP. Finally, three repeated NLS sequences were added in the C terminus of E-Cas9 RNP, forming the plasmid named that would be expressed to the engineered Cas9 RNP in this study.

Expression and purification of recombinant Cas9 RNPs. The *E.coli* Rosetta (DE3) competent cells were transformed with the plasmid Ptac-cas9-T7sgRNA or E-Cas9 RNP-3NLS to produce the Cas9 RNPs and engineered Cas9 RNPs, respectively. The selected monoclones were inoculated in Luria-Bertani (LB) broth containing 100 mg/ml of ampicillin at 37^{o}C overnight. The cells were then diluted 1:100 into the same growth medium and grown at 37^{o}C to $OD_{600} \approx 0.6$. Next, the culture was incubated at

4°C for 30 minutes, followed by adding isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 mM for induction of Cas9 RNPs at 18 °C. After 16 h, the cells were collected, re-suspended in lysis buffer (20 mM Tris-HCl. pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol), broken by sonication, and purified on Ni-NTA resin followed by another G75 gel filtration column. The resulting Cas9 RNPs were desalted and concentrated to ~2 mg/ml by Amicon Ultra centrifugal filters (Millipore, 100-KDa) and stored at -20°C in storage buffer (10 mM Tris-Cl, pH 7.4, 500 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol).

***In vitro* endonuclease activity test of Cas9 RNPs.** For *in vitro* endonuclease activity assay, the purified Cas RNPs from *E. coli* were directly applied to digest the plasmids containing target dsDNA sequences. The digestion was typically carried out in a 10 μL volume of reaction mixture, composed of 1 μL 10 x buffer 3.1 (NEB), 200 ng Cas9 RNPs, 300 ng plasmid, at 37°C for 30 minutes followed by termination of the reaction at 80°C for 10 minutes. The cleaved DNA fragmentation was evaluated by 2% agarose gel electrophoresis.

Synthesis of RFP@CS nanoparticles. Chitosan (5 g) was firstly dissolved and protonated in 25 ml 1 M HCl. Then 100-150 ml of absolute ethanol was added to precipitate protonated chitosan oligosaccharide (COS+). After centrifugation, lyophilization and grinding, the protonated COS+ powder was obtained. For each preparation, RFP (10 mg) was dissolved in 20 ml of PBS (20 mM, pH 7.4). The COS+ was added, and stirred at room temperature for 30 min. After that, glutaraldehyde (Glu, 2.5%) was added as the cross-linker. The molar ratio of protein: amino glucose units of COS+: Glu was 1: 2000: 700. The polymerization continued for 12 h at room temperature. At last, the chitosan coated RFP nanoparticles were purified and concentrated by ultracentrifuge (Millipore MWCO 100-kDa ultrafilter). The RFP@CS NPs could be stored either as stable concentrated suspension, or in solid powder obtained by lyophilization.

Particle size (DLS and TEM) and zeta potential analysis. Transmission electron microscope (TEM) images of RFP@CS with and without Cas9 RNPs and ssDNA were obtained with a JEM 200 CX TEM (JEOL, Japan), and diameters of 360 particles from the images were counted. The particle size of RFP@CS and Cas9 RNP-ssDNA-RFP@CS nanocomplex in aqueous suspension were also examined using a nanosizer (dynamic light scattering [DLS], Malvern Nano ZS90, Malvern, UK) at 25°C. Meanwhile, the zeta potentials of RFP@CS, Cas9 RNP, Cas9 RNP-ssDNA-RFP@CS nanocomplex and Cas9 RNP-RFP@CS nanocomplex were also measured. Each sample was prepared and incubated for a few minutes to form particles, then transferred to the capillary cell. An equilibration time ranging from two to five minutes was needed to optimize the DLS measurements and collect accurate DLS data.

Ultraviolet-visible (UV-Vis) spectra and fluorescence spectra. The absorbance spectra of RFP@CS, FITC-Cas9 RNP and FITC-Cas9 RNP-RFP@CS nanocomplex were measured by an ultraviolet-visible spectrophotometer (HITACH U3900). Fluorescence spectra changes of FITC labelled Cas9 RNP once loaded to RFP@CS NPs were determined with fluorescence spectroscopy (HITACHI F-4600) with an excitation at 490 nm. Loading capacity of Cas9 RNP onto RFP@CS was determined by the decreases of fluorescence intensity for Cas9 RNP in the supernatant before and after incubation with RFP@CS, using an Amicon Ultra centrifugal filters (Millipore, 300-KDa).

Fluorescence microscopy and flow cytometry analysis. The delivery of Cas9 RNP into cells by RFP@CS were monitored by the fluorescence microscopy (Olympus IX73). In a typical experiment, we co-delivered 2 μg Cas9 RNP and 1 μg ssDNA donor by 50 μg RFP@CS into HeLa cells in 12-well plate for five hours, and then measured their fluorescence images using different fluorescence channels. The transfection efficiency was quantified by detection of FITC-Cas9 RNP inside the cells using BD LSRFortessa X-20 and Guava easyCyte. Moreover, the mechanism of cellular uptake was investigated by pre-treatments with methy-β-cyclodextrim (MβCD, 7.5 mg/ml),

chlorpromazine (CPM, 1.5 μg/ml), amiloride (0.6 mg/ml) and low temperature (4 oC) for 30 min, followed by the incubation with FITC-Cas9 RNP-RFP@CS nanocomplex for 2 h. To further quantify the intracellular fluorescence intensity, all cells were collected and lysed with RIPA lysis buffer for 0.5 h on ice, followed by the measurement of the fluorescence intensity.

Detecting the efficiency of HDR-mediated genome editing. For illustrating in vivo homology dependent repair (HDR) efficiency of the purified Cas RNPs, we adopted a BFP-expressing HEK293 reporter cell line according to the protocols. To edit BFP to GFP, the 70 nt ssDNA donor and Cas9 RNPs were co-delivered by RFP@CS NPs or lipofectamine CRISPRMAX (ThemoalFisher). The HDR efficiency was calculated from GFP cell counts/total cell counts.

Detecting the efficiency of NHEJ-mediated genome editing. After delivery of Cas9 RNP by RFP@CS NPs for 5 h, different cells including HEK293T, RAW264.7 and HeLA cells were washed and replaced with DMEM (with 10% FBS and 1% antibiotics) and then allowed to grow for another 48 h. Then, cells were harvested to extract genomic DNA using a QuickExtract genomic DNA isolation kit (Omega). Indel assays were performed using T7 endonuclease-I according to standard protocol.

Cell viability assays. The prepared RFP@CS NPs were dispersed in deionized PBS buffer (pH 7.4) to prepare the stock solution (10 mg/ml). The stock solution was diluted to test concentrations with culture medium, and loaded them with Cas9 RNPs and ssDNA just prior to cell exposure. HEK293 cells, RAW264.7 cells and HeLa cells were cultured in DMEM (high glucose) supplemented with penicillin-streptomycin and fetal bovine serum (FBS) (10% v/v) (Gibco, USA) at 37°C in humidified air with 5% CO_2. Cell viability was determined with WST-8 cell counting kit (CCK-8, Dojindo Molecular Technologies, Japan).

Supporting Information for Experimental Procedures

The sequence of ssDNA donor used in this study is:

5'GTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGGGTGGTCACGAGG GTGGGCCAGGGCACG-3'

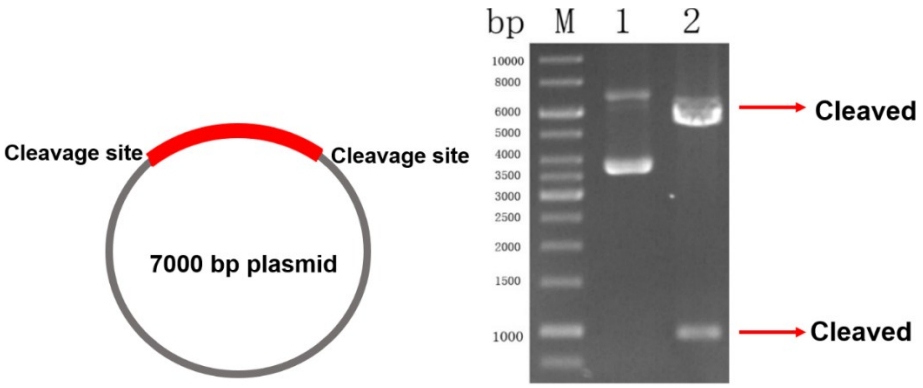

Fig. S1 Cleavage of 0.1 pmol plasmid shown in the left scheme (right panel, lane 1) by addition of 1.2 pmol Cas9 RNPs (right panel, lane 2) at 37°C for 1 hours. There are two identical Cas9 recognition cleavage sites. Therefore, cleavage of the plasmid by Cas9 RNP resulted in production of one 6000 bp DNA fragment and one 1000 bp DNA fragment

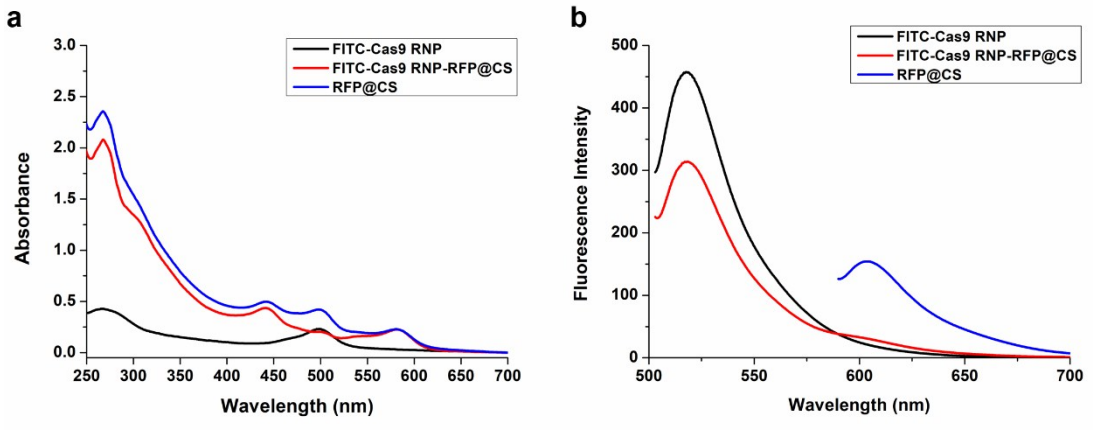

Fig. S2 (a) Absorbance and (b) fluorescent emission spectra of bare RFP@CS (blue), FITC-Cas9 RNP (black) and FITC-Cas9 RNP-RFP@CS (red). The excitation wavelength is 490 nm for FITC-Cas9 RNP and 586 nm for RFP@CS, respectively.

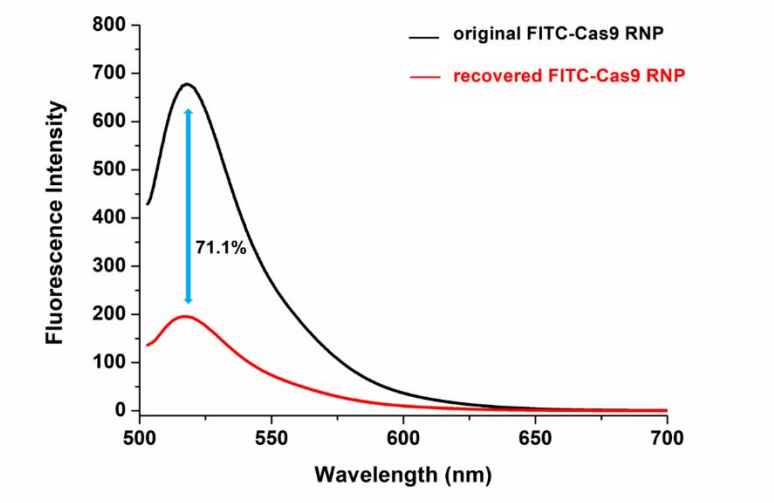

Fig. S3 Fluorescence spectra of original FITC-Cas9 RNP and recovered FITC-Cas9 RNP from spin filtration. The decreased fluorescence intensity indicated that 71.1% FITC-Cas9 RNP was loaded on the surface of RFP@CS NPs.

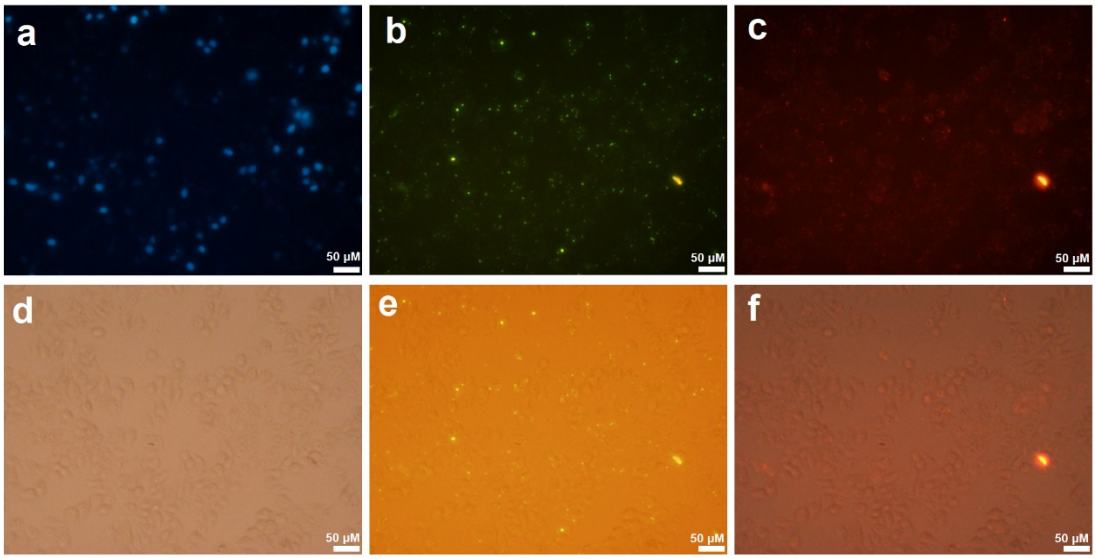

Fig. S4 Cellular delivery of FITC labelled Cas9 RNPs and ssDNA donor by RFP@CS into HeLa cells. Fluorescence microscopy images of FITC-Cas9 RNP/ssDNA/RFP@CS after incubation with HeLa cells for 5 h. (a) blue color represents the cell nucleus that were stained with Hoechst; (b) green color represents the FITC- Cas9 RNPs; (c) red color represents the RFP@CS; (d) the bright-field; e) the overlap of b and d; (f) the overlay of c and d.

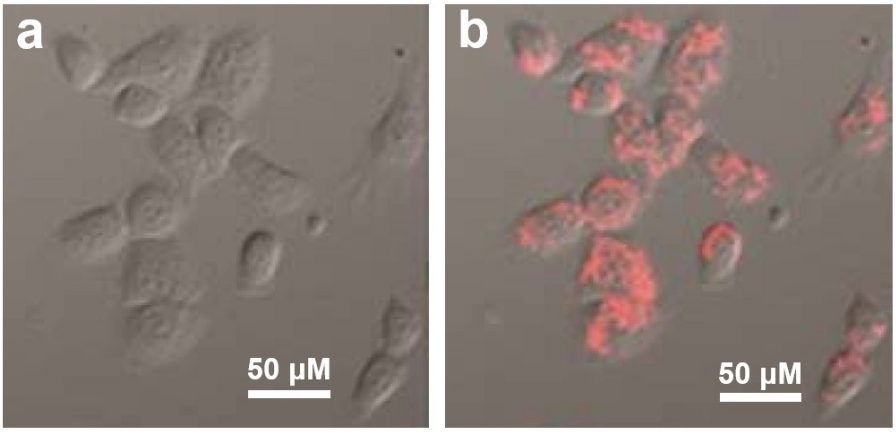

Fig. S5 Cellular delivery of Cas9 RNP/ssDNA by RFP@CS. Confocal microscopy imagines of RFP@CS (red) delivering Cas9 RNP/ssDNA after 5 h incubation with HeLa cells (excited at 534 nm). (a) Bright field; (b) Red Channel.

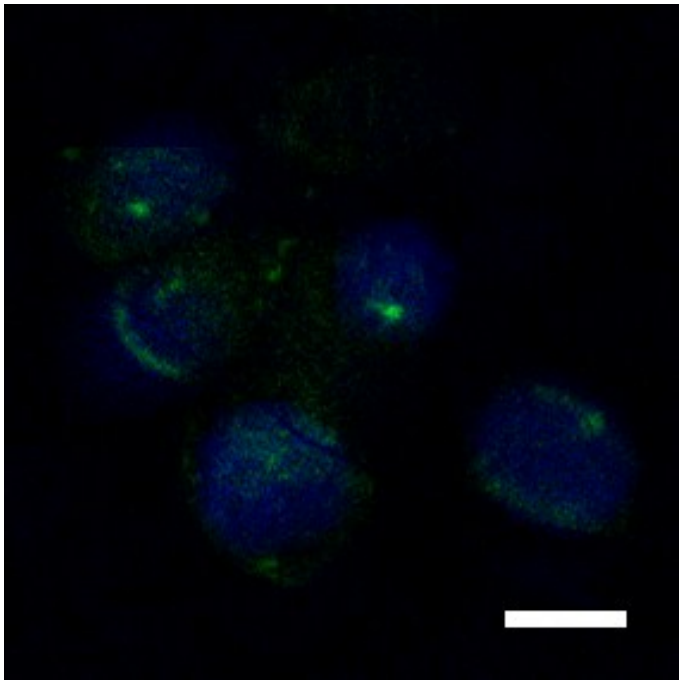

Fig. S6 Confocal microscopy image of RFP@CS delivering FITC-Cas9 RNP/ssDNA after 12 incubation with HeLa cells. The blue channel and green channel are merged. The cell nuclei are stained with Hoechst. Scale bar is 20 μM.

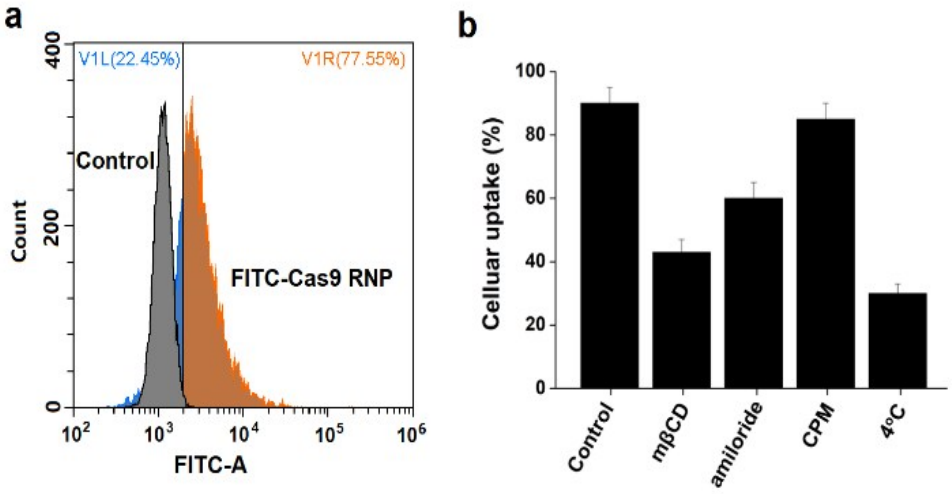

Fig. S7 (a) The low cytometry results of HeLa cells including control (grey) and samples transfected by FITC-Cas9 RNPs and ssDNA (blue + orange) using RFP@CS as the carrier vector. (b) Inhabitation of cellular uptake of FITC-Cas9 RNP-RFP@CS nanocomplexes by various inhibitors and low temperature.

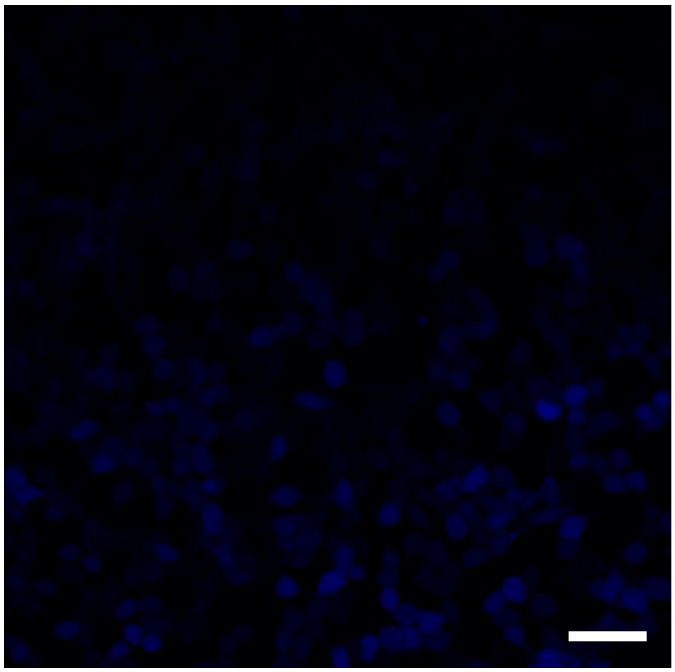

Fig. S8 Co-delivery of Cas9 RNP and a 70 nt ssDNA donor by RFP@CS into BFP-expressing H293T cells (before the delivery). Scale bar is 100 μM. The image was recorded by a confocal microscope (Zeiss LSM 710).

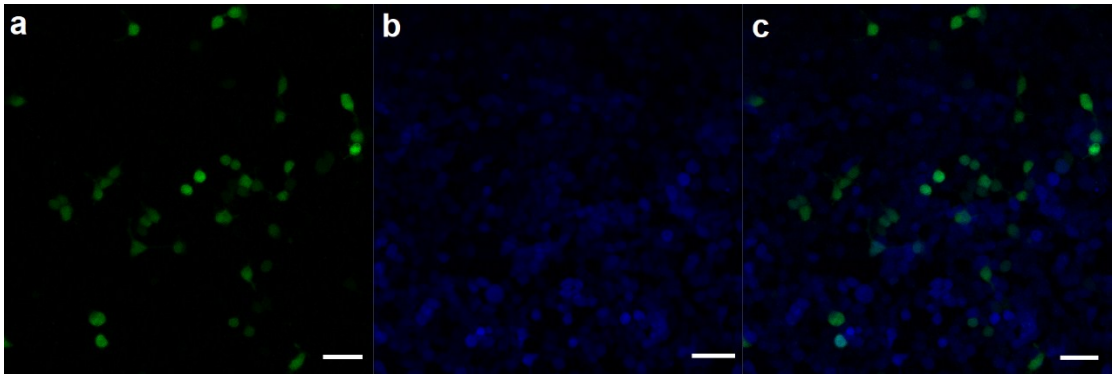

Fig. S9 Co-delivery of Cas9 RNP and a 70 nt ssDNA donor by RFP@CS into BFP-expressing H293T cells to induce HDR which transferred BFP into GFP. (a) The green color channel; (b) blue color channel; (c) the overlap of a and b. Scale bar is 100 μM. The images were recorded by a confocal microscope (Zeiss LSM 710).

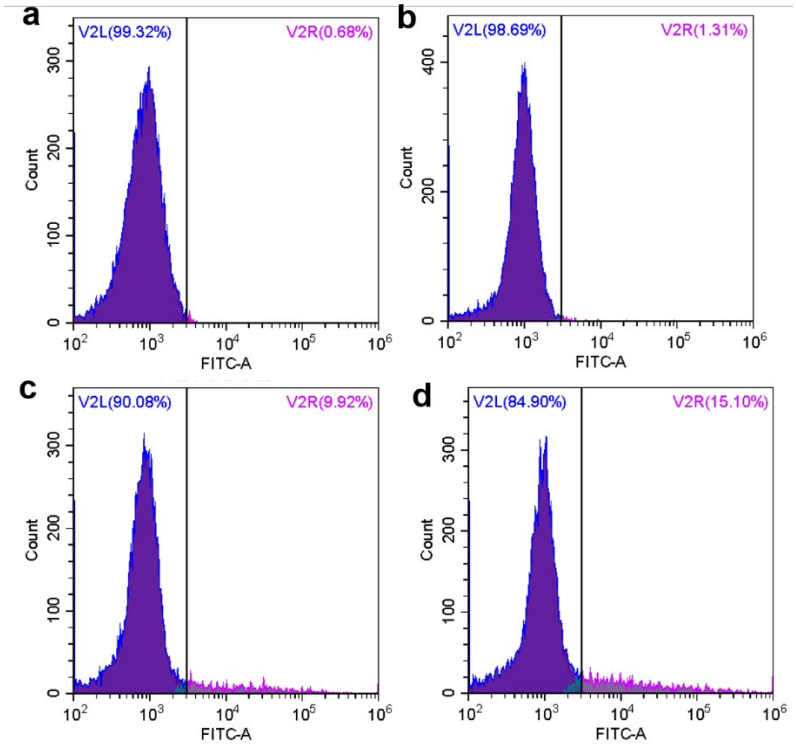

Fig. S10 Measurements of the HDR efficiency by the flow cytometry data. (a) HEK293 cells; (b) transfection of sgRNA; (c) transfection of Cas9 RNP and gRNA by Lipofectamine CRISPRMAX; (d) transfection of Cas9 RNP and gRNA by RFP@CS NPs.

Fig. S11 Addition of Cas9 RNP (without chitosan) and a 70 nt ssDNA donor into BFP-expressing H293T cells. The fluorescence microscopy under green channel was shown, which indicates that no HDR editing when using Cas9 RNP only.

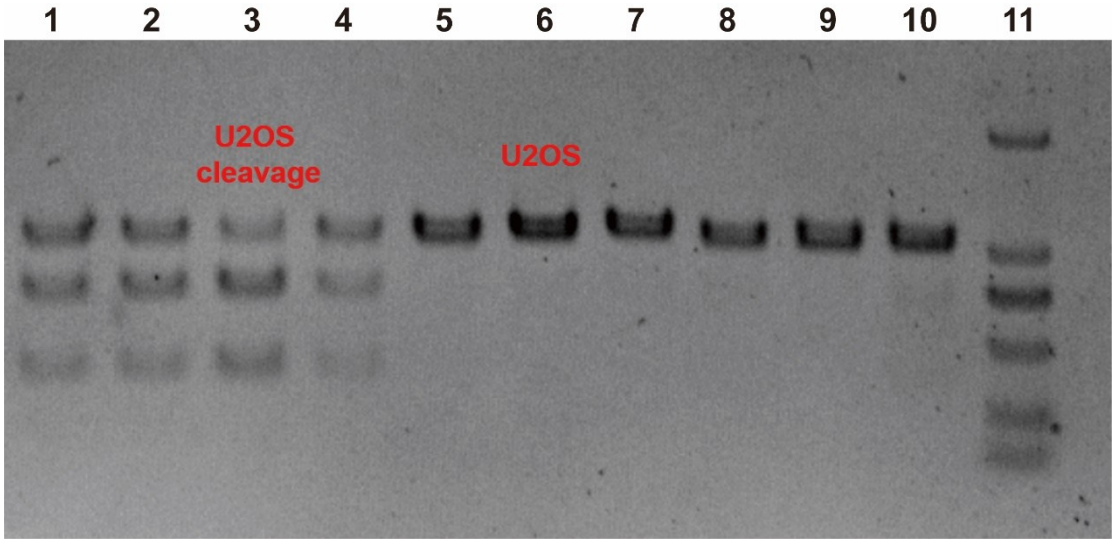

Fig. S12 The full labeled gel for drawing the results of T7 endonuclease-I assay regarding U2OS cell line (lane 1 to lane 8) in Fig. 4C (marked in red color). lane 1 to lane 4 contain the target *PRDX4* PCR fragments after cleavage by T7 endonuclease-I; lane 5-8 contain the target *PRDX4* PCR fragments; lane 9 is the target *PRDX4* PCR fragment derived from A549 cell line; lane 10 is the target *PRDX4* PCR fragment after cleavage by T7 endonuclease-I derived from A549 cell line; line 11 is the protein ladder.

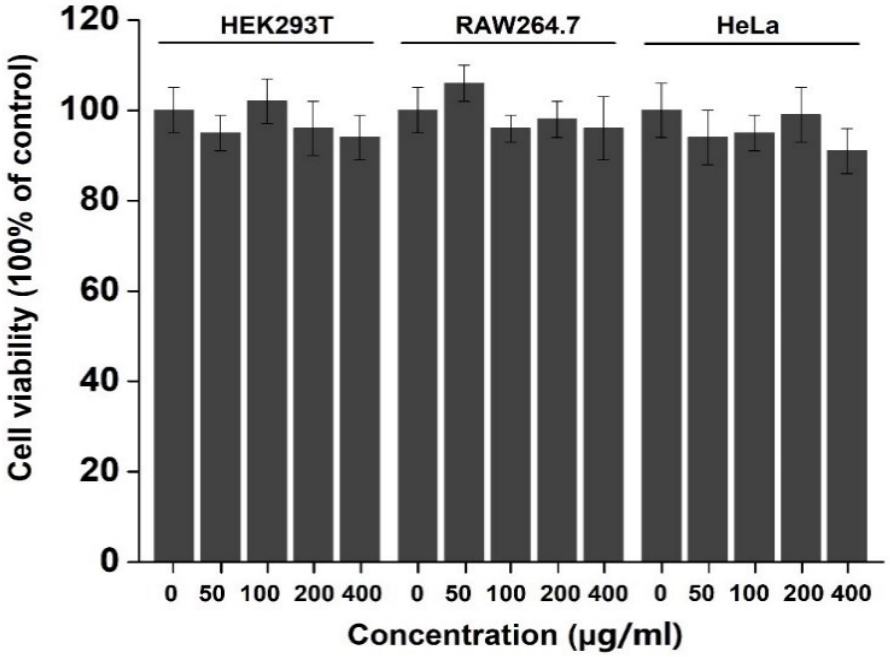

Fig. S13 Cell viability of HEK293T, RAW264.7 and HeLa cells exposed to different doses of Cas9 RNP-ssDNA-RFP@CS nanocomplexes.