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A DNAzyme-Mediated Genetically Encoded Sensor for Ratiometric Imaging of Metal Ions in Living Cells

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Experimental Procedures

Materials. Oligonucleotides were all purchased from Integrated DNA Technologies (Coralville, US). The plasmids (Clover2-N1 #54537, mRuby2-N1 #54614) were purchased from Addgene as transformed bacteria stab, then extracted with Plasmid EndoFree Maxi Kit (Qiagen). ^{32}P labeled γ-ATP for DNA radiolabeling was obtained from Perkin-Elmer. The T4-polynucleotide kinase (PNK) was purchased from New England Biolabs. Lipofectamine 3000 was obtained from Thermo Fisher Scientific. Calcimycin, DEPC, magnesium chloride, and sodium chloride were purchased from Sigma. All buffers, metal ions, and gel stock solutions were prepared with molecular biology grade water purchased from Fisher.

Instrumentation. The radioactive gels and non-radioactive gels were imaged by Molecular Dynamics Storm 430 phosphorimager (from Amersham Biosciences) and Bio-Rad Gel Doc 2000 imaging system correspondingly. Cell images were taken using a Zeiss LSM 880 or Zeiss LSM 710 confocal microscope at 10× or 63× magnification. Flow cytometry data were obtained using a BD FACS Canto system.

Polyacrylamide Gel Electrophoresis (PAGE) Gels Analysis. The RNA substrate was labeled with γ-ATP by adding PNK enzyme (0.5 μL), 10× PNK buffer (1 μL) and γ-ATP (4 μL) to RNA substrate (4.5 μL 10 μM). Subsequently, the solution was incubated in a PCR machine (37°C for 2h, then 65°C for 20min). Various concentrations of DNAzymes or mutant DNAzymes (Dz10M, Dz9M, and Dz8M) were incubated with 200 nM γ-ATP labeled RNA substrate to reach the certain DNAzyme (or mutant DNAzymes): substrate ratios. The reactions were carried out in Tris-HCl buffer (50 mM Tris, 150 mM NaCl, 30 mM $MgCl_2$, pH 7.4) at 37°C for 60 min, then the reactions were quenched by stop-solution (50 mM EDTA, 8 M Urea, 1x TBE, 0.05 % xylene cyanol and 0.05 % bromophenol blue). Then the samples were analyzed by 15% denaturing PAGE gel for 30 min at 26 W with 0.3% DEPC treated TBE running buffer. The result was imaged by Molecular Dynamics Storm 430 phosphorimager then quantified by Image J.

The enzymatic activity of DNAzymes was quantified by the following steps. First, a rectangular box surrounding the full-length substrate band was selected on the gel picture, and the intensity of this rectangular region of interest was quantified by the Image J software. Second, all the gel images will have some background signal, so a rectangular box with the same area right above the region of interest containing the full-length band (in the same lane) was selected, and the intensity of this background region was measured. Third, the measured background intensity was subtracted form the measured band intensity to calculate the true band intensity ($I_{full-length}$). Fourth, the previous steps were repeated for each full-length band and cleaved band on the gel. The intensity of bands corresponding to full-length substrates ($I_{full-length}$) and cleaved substrates ($I_{cleaved}$) were calculated. The enzymatic activity is calculated by the portion of substrate that is cleaved in each lane following the formula $I_{cleaved} / (I_{full-length} + I_{cleaved})$. The higher this ratio is, the more effective for the DNAzyme to cleave substrate RNAs, interpreted as higher enzymatic activity.

The non-radioactive PAGE was conducted by incubating 200 nM Dz10 with 400 nM RNA substrate in Tris-HCl buffer (50 mM Tris, 150 mM NaCl, pH 7.4) with 0, 10, 20, 40 mM $MgCl_2$ at 37°C for 60 min. The samples were analyzed with 15% native gel at 5w for 25min. After electrophoresis, the gel was stained by ethidium bromide then imaged.

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Cleavage Site Prediction. The cleavage site was selected according to the preferred cleavage site selection strategy for efficient DNAzymes.[1] RNAfold prediction of the secondary structure of the $mRNA_{CloverFP}$ was conducted at 37°C. The computation was done using RNAfold from the Vienna RNA Suite and parameters from Andronescu model (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

Cell Culture, DNAzyme, and Plasmid Transfection. HeLa cells (from ATCC) were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin, and were incubated in a humidified 5% CO_2 incubator at 37°C. The cells were seeded in 12 well-plates or optical dishes 24h before transfection. DNAzyme, CloverFP, and RubyFP plasmids were co-transfected into HeLa cells with Lipofectamine 3000 following the protocol provided by the manufacturer.

Imaging Intracellular Mg^{2+} or Zn^{2+}. 3.0×10^4 HeLa cells were cultured in 8 wells optical dish for 24 h. Then cells were transfected with 300 ng DNAzyme, 100 ng CloverFP, and 100 ng RubyFP plasmids. After incubation for 6h, the medium was replaced by fresh DMEM containing 2 μM calcimycin and different concentrations of Mg^{2+} or DMEM containing 30 μM Zn^{2+}, then the cells were incubated for another 18 h. The cells were washed 3 times with PBS prior to confocal microscopy imaging. Images were taken using a Zeiss LSM 880 high-resolution confocal microscope at 10× magnification. Fluorescence of CloverFP was obtained by excitation at 488 nm and collected over 493-563 nm. Fluorescence of RubyFP was obtained by excitation at 561 nm and collected over 603-752 nm. Quantification of the image was performed by ZEN Blue software.

Localizing DNAzyme in cells. The cells were transfected with 300 ng Cy5-DNA and 100 ng CloverFP plasmids. After incubation for 6 h or 24 h, the cells were washed 3 times with PBS and stained with Lysotracker Green for imaging. Images were taken using a Zeiss LSM 710 high-resolution confocal microscope at 63× magnification. Fluorescence of Lysotracker and CloverFP was obtained by excitation at 488 nm and collected over 497-549 nm. Fluorescence of Cy5-DNA was obtained by excitation at 633 nm and collected over 629-726 nm.

Flow Cytometry Detection. 2.0×10^5 HeLa cells were cultured in a 12 well-plate for 24 h to reach 70-90% confluency. Then cells were transfected with 600 ng DNAzyme, 200 ng CloverFP, and 200 ng RubyFP plasmids. After incubation for 6h, the medium was replaced by fresh DMEM containing 2 μM calcimycin and different concentrations (0, 20, 40 mM) of Mg^{2+}, then the cells were incubated for another 18 h. Then cells were washed with PBS and detached from the 12-well plate by 0.05% trypsin. The suspended cells were centrifuged and dispersed in PBS for flow cytometry. Flow cytometry was conducted using a BD FACS Canto system. In total, 1.0×10^4 cells were counted for each sample. Fluorescence of CloverFP was obtained by excitation at 488 nm and collected with a 530 ± 30 nm filter. Fluorescence of RubyFP was obtained by excitation at 561 nm and collected with a 610 ± 20 nm filter. The results were analyzed with FCS Express 6 Flow Cytometry Software.

For the mutant DNAzymes study, 600 ng Dz10TCloverFP, Dz10TMCloverFP, Dz10HCloverFP or Dz10HMCloverFP was co-transfected with 200 ng CloverFP and 200 ng RubyFP plasmids in 12 well-plate. After incubation for 6h, the medium was replaced by fresh DMEM containing 2 μM calcimycin and different concentrations (0, 20 mM) of Mg^{2+}, then the cells were incubated for another 18 h. Subsequent flow cytometry detection was conducted.

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Table S1. DNA and RNA Sequences used in this study. "*" means phosphorothioate, which is introduced to protect the DNA from degradation in cells. "inT" means inverted T, which is introduced to protect the DNA from degradation in cells. The underlined sequences indicate the catalytic domain of the DNAzyme.

Name	Sequence from 5' to 3'
RNA Substrate	rGrGrU rGrGrU rCrArU rGrGrA rArGrG rUrUrC rGrGrU rCrArA rCrGrG rCrCrA
12Dz	CC GTT GAC CGA A GGCTAGCTACAACGA C TTC CAT GAC CA inT
11Dz	C GTT GAC CGA A GGCTAGCTACAACGA C TTC CAT GAC C inT
10Dz	GTT GAC CGA A GGCTAGCTACAACGA C TTC CAT GAC inT
9Dz	TT GAC CGA A GGCTAGCTACAACGA C TTC CAT GA inT
8Dz	T GAC CGA A GGCTAGCTACAACGA C TTC CAT G inT
10DzM	GTT GAC CGA A GGCTA***C***CTACAACGA C TTC CAT GAC inT
9DzM	TT GAC CGA A GGCTA***C***CTACAACGA C TTC CAT GA inT
8DzM	T GAC CGA A GGCTA***C***CTACAACGA C TTC CAT G inT
$Dz10T_{CloverFP}$	T CCA GCT CGA GGCTAGCTACAACGA CAG GAT GGG C inT
$Dz10TM_{CloverFP}$	T CCA GCT CGA GGCTA***C***CTACAACGA CAG GAT GGG C inT
$Dz10H_{CloverFP}$	GCGCGCG AAA CGCGCGC GGG T CCA GCT CGA GGCTAGCTACAACGA CAG GAT GGG C GTA CGCGCGC AAA GCGCGCG
$Dz10HM_{CloverFP}$	GCGCGCG AAA CGCGCGC GGG T CCA GCT CGA GGCTA***C***CTACAACGA CAG GAT GGG C GTA CGCGCGC AAA GCGCGCG
Cy5-DNA	Cy5- ATA GTT TCT CCG AGC CGG TCG AAA CTT CTC TAC C*T*G* C*A*A
$Cy5\text{-}Dz10T_{CloverFP}$	Cy5- T CCA GCT CGA GGCTAGCTACAACGA CAG GAT GGG C inT
$Cy5\text{-}Dz10H_{CloverFP}$	Cy5-GCGCGCG AAA CGCGCGC GGG T CCA GCT CGA GGCTAGCTACAACGA CAG GAT GGG C GTA CGCGCGC AAA GCGCGCG
10Zn-GG	ACCGA ACCTT T CCG AGC CGG TCGAA ATG ACC ACC T
10ZnR-1	ACC GAA CCT TT CCG AGC CGG TCGAA ATG ACC ACC T inT
10ZnR-2	GTT TGA GTT CT CCG AGC CGG TCGAA ATG TAC GGA T inT
10ZnR-3	AGT ACG GCT GT CCG AGC CGG TCGAA ATA CAT GAA C inT
10ZnR-4	GAC GAC TCC AT CCG AGC CGG TCGAA ATC TTC GTA T inT
10ZnR-5	AAC GAG ACA GT CCG AGC CGG TCGAA ATC CTC AAG G inT
10ZnR-6	CAT CAC GGG AT CCG AGC CGG TCGAA ATT GGA GGG A inT
10ZnR-7	CCT CAG ACC AT CCG AGC CGG TCGAA ATC TGC TGG A inT
10ZnR-8	ACT TTC AGT GT CCG AGC CGG TCGAA ATA TGA GTG T inT
10ZnR-9	ATG GCC ACC AT CCG AGC CGG TCGAA ATC AAC TTT C inT
10ZnC	TCC AGC AGG AT CCG AGC CGG TCGAA ATG TGA TCG C inT

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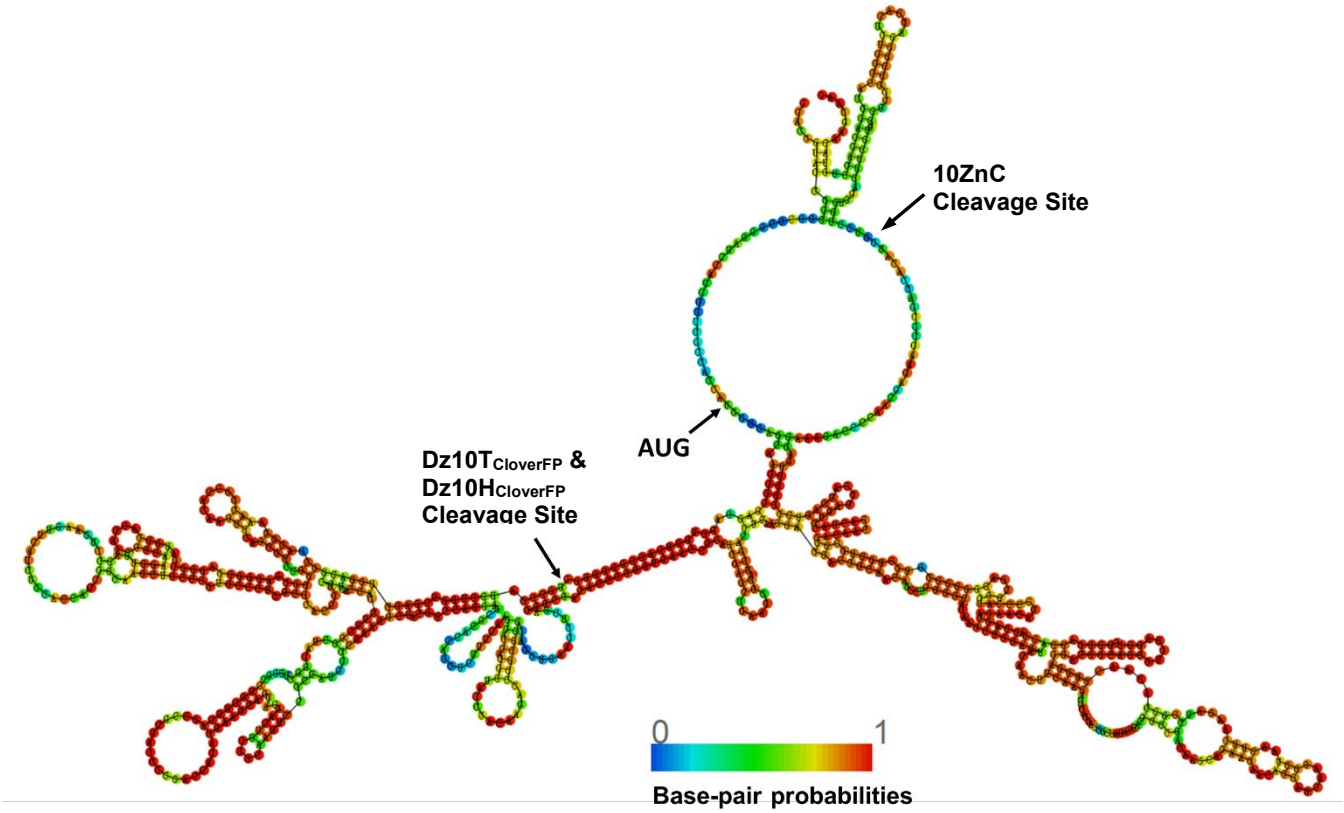
Sequence of $mRNA_{CloverFP}$ (5′ to 3′)

The start codon and cleavage site are marked as green and red, correspondingly.

cgacgguaccgcgggcccgggauccaccggucgccaccauggugagcaagggcgaggagcuguucaccgggguggugcccauccuggu(Dz10$T_{CloverFP}$ and Dz10$H_{CloverFP}$)cgagcuggacggcgacguaaacggccacaaguucagcguccgcggcgagggcgagggcgaugccaccaacg
gcaagcugacccugaaguucaucugcaccaccggcaagcugcccgugcccuggcccaccucgugaccaccuucggcuacggcguggccugcuu
cagccgcuaccccgaccacaugaagcagcacgacuucuucaaguccgccaugcccgaaggcuacguccaggagcgcaccaucucuucaagga
cgacgguaccuacaagacccgcgccgaggugaaguucgagggcgacacccuggugaaccgcaucgagcugaagggcaucgacuucaaggagg
acggcaacauccuggggcacaagcuggaguacaacuucaacagccacuacgucuauaucacggccgacaagcagaagaacagcaucaaggcu
aacuucaagauccgccacaacguugaggacggcagcgugcagcucgccgaccacuaccagcagaacaccccaucggcgacggccccgugcug
cugcccgacaaccacuaccugagccaucaguccgcccugagcaaagaccccaacgagaagcgcgaucacaugg(10ZnC)uccugcuggaguu
cgugaccgccgccgggaucacucucggcauggacgagcuguacaaguaag



Sequence of $mRNA_{RubyFP}$ (5′ to 3′)

gggcccgggauccaccggucgccaccaugugucuaagggcgaagagcugaucaaggaaaauaugcguaugaaggugucaugg(10ZnR-1)aaggguucggucaacggccaccaauucaaaugcacaggugaaggagaaggcaauccguacaugg(10ZnR-2)gaacucaaaccaugaggaucaaagucaucgagggaggaccccugccauuugccuuugacauucuugccacgucguucauguauugg(10ZnR-3)cagccguacuuuuaucaaguacccgaaaggcauuccugauuucuuuaaacaguccuuuccugaggguuuuacuugggaaagaguuacgagauacgaagaugg(10ZnR-4)uggagucgucaccgucaugcaggacaccagccuugaggaugg(10ZnR-5)cugucucguuuaccacguccaagucagagggguaaacuuuccccuccaaugg(10ZnR-6)ucccgugaugcagaagaagaccaaggguugggagccuaauacagagaugauguauccagcagaugg(10ZnR-7)uggucugaggggauacacucauauugg(10ZnR-8)cacugaaaguugaugg(10ZnR-9)uggugggccaucugucuugcucuuucguaacaacuuacaggucaaaaaagaccgucgggaacaucaagaugcccgguauccaugccguugaucaccgccuggaaaggguuagaggaaagugacaaugaaauguucguaguacaacgcgaacacgcaguugccaaguucgccgggcuuggugguggggauggacgagcuguacaaguaagcggccgcgacucuag

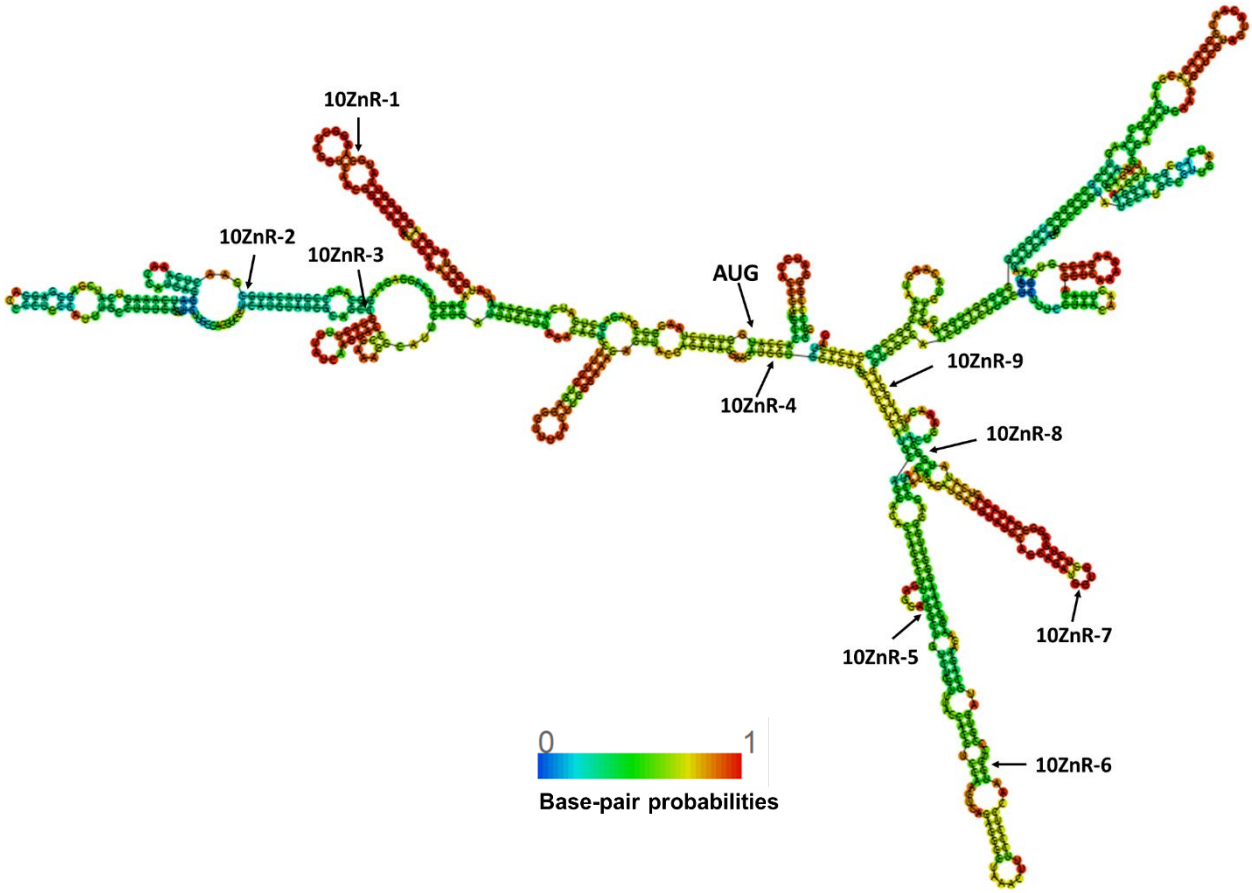


Figure S1. The predicted secondary structure of $mRNA_{CloverFP}$ and $mRNA_{RubyFP}$ with potential 8-17 DNAzyme cleaving sites. Color bar indicates base pair probability. The location of the cleavage site and start codon are marked.

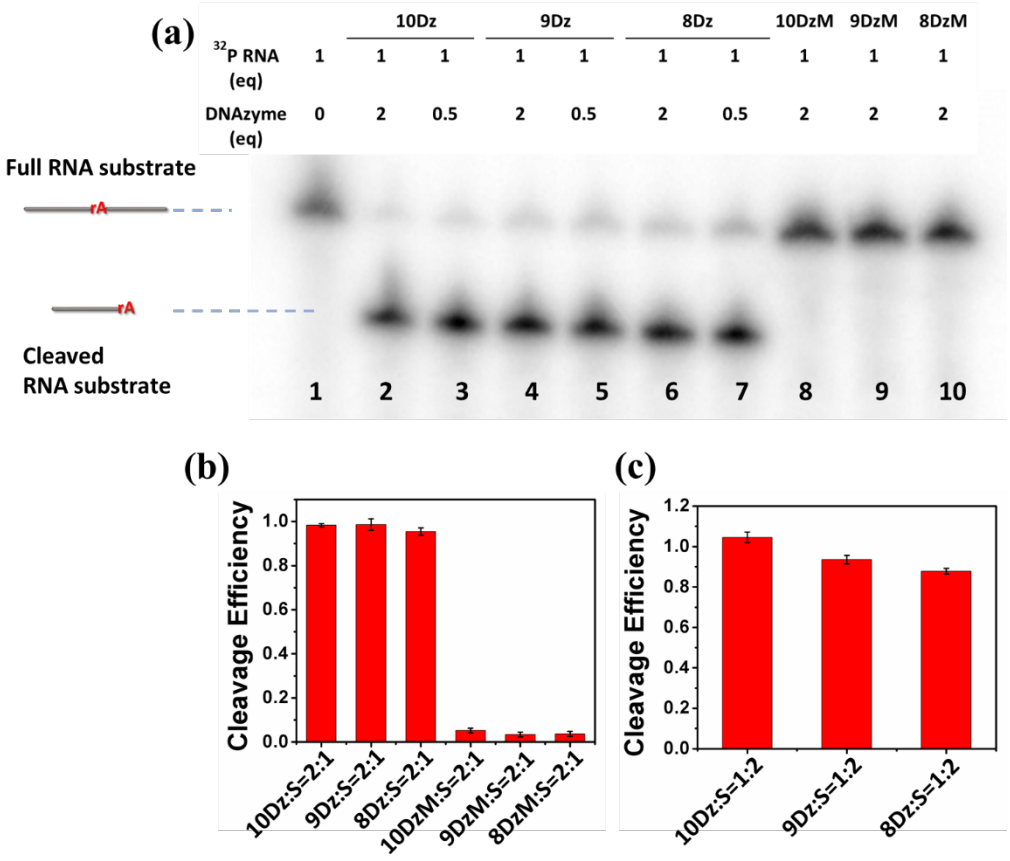


Figure S2. (a) Gel electrophoresis analysis of the RNA cleavage efficiency using 10Dz, 9Dz, 8Dz and their mutants (10DzM, 9DzM, 8DzM) with the DNAzyme: substrate ratio of 2:1 (lane 2, 4, 6) or 1:2 (lane 3, 5, 7), DNAzyme mutant: substrate ratio of 2:1 (Lane 8 to lane 10). (b) The quantification of the gel electrophoresis analysis with the DNAzyme (or their mutants) to RNA substrate ratio of 2:1 or (c) 1:2. Data shown in mean and S.D.

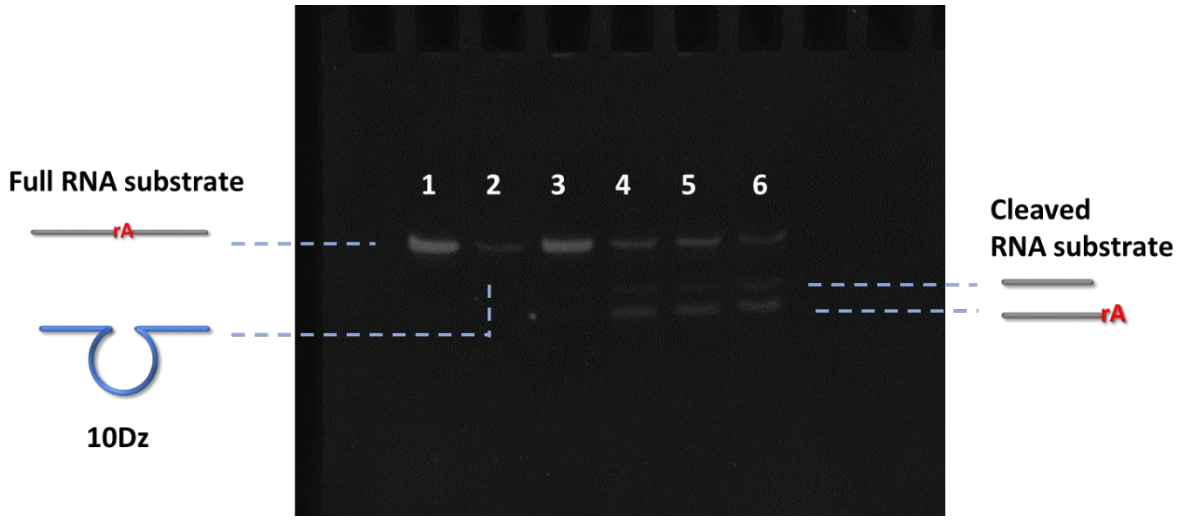


Figure S3. Gel electrophoresis analysis of the RNA cleavage efficiency using 10Dz in different concentration of Mg^{2+}. RNA substrate (lane 1), 10Dz (lane 2), RNA substrate and 10Dz in 0, 10, 20, 40 mM Mg^{2+} (lane 3 to lane 6).

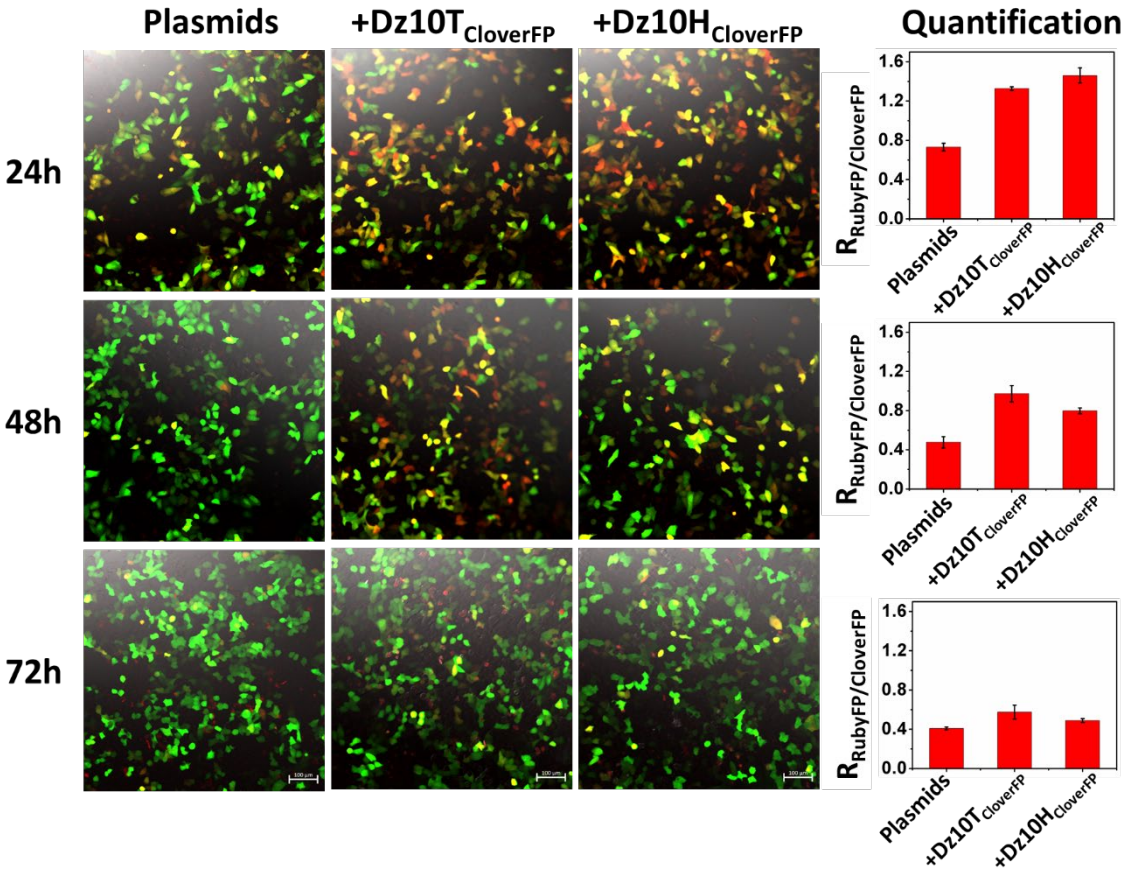


Figure S4. The confocal imaging of cells transfected with $Dz10T_{CloverFP}$ or $Dz10H_{CloverFP}$ and plasmids for 24, 48 and 72 hours. Scale bar: 100 μm. Bar graphs showed the ratio of RubyFP / CloverFP value in mean and S.D.

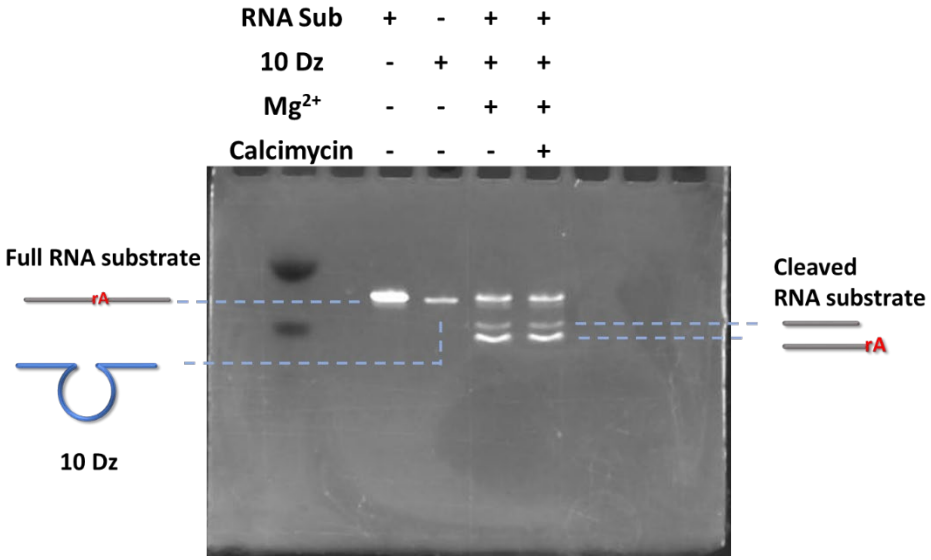


Figure S5. Gel electrophoresis analysis of the RNA cleavage efficiency in the present of calcimycin. 400 nM RNA substrate, 200 nM 10Dz, 30 mM MgCl2, and 2 μM calcimycin in Tris-HCl were used to conduct this experiment.

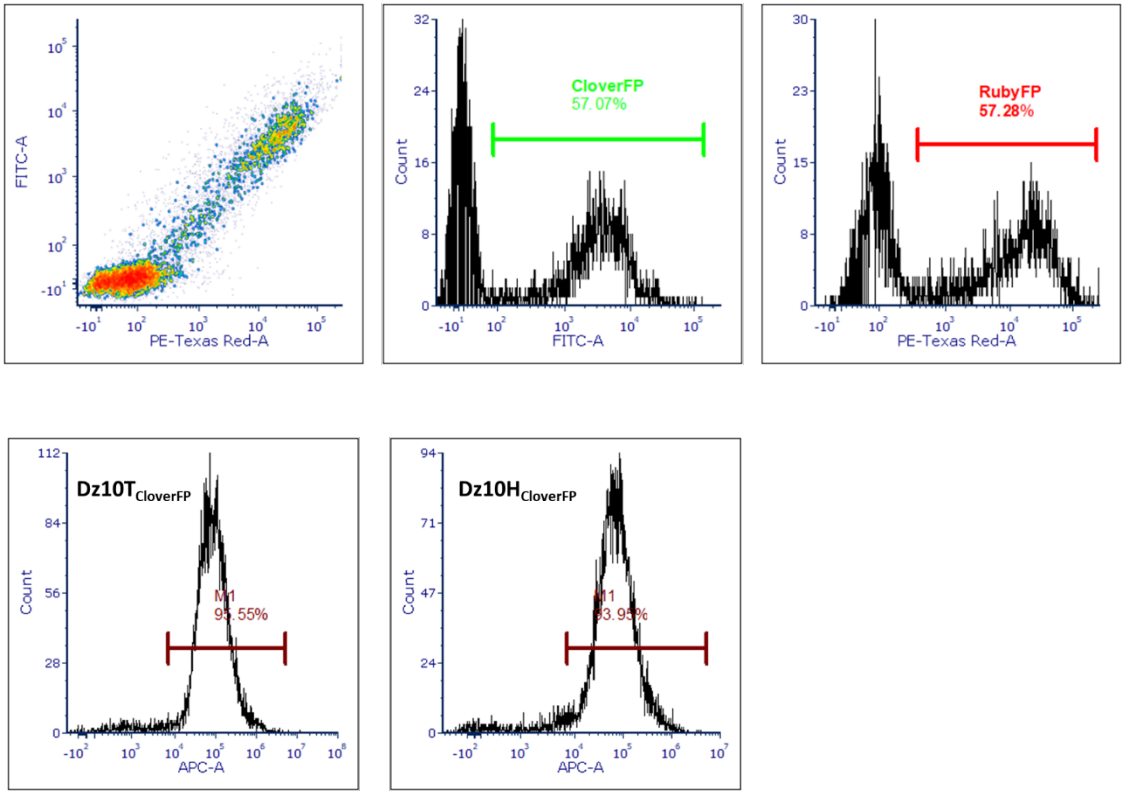


Figure S6. The co-transfection efficiency of plasmids bearing CloverFP and RubyFP, and the transfection efficiency of $Dz10T_{CloverFP}$ and $Dz10H_{CloverFP}$.

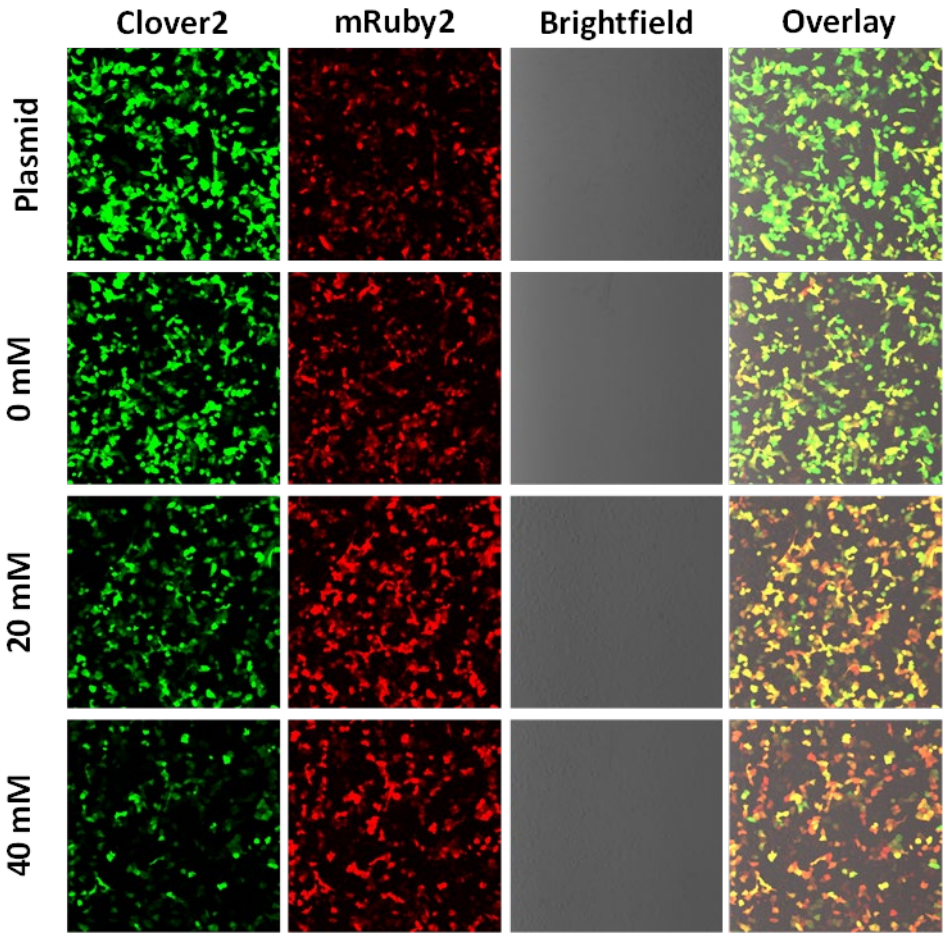


Figure S7. Confocal imaging of the cells expressing CloverFP (green channel) and RubyFP (red channel). The cells were transfected with plasmids only (first row) or with plasmids and CDz10H$_{CloverFP}$ in different concentration of Mg^{2+} (second to fourth rows).

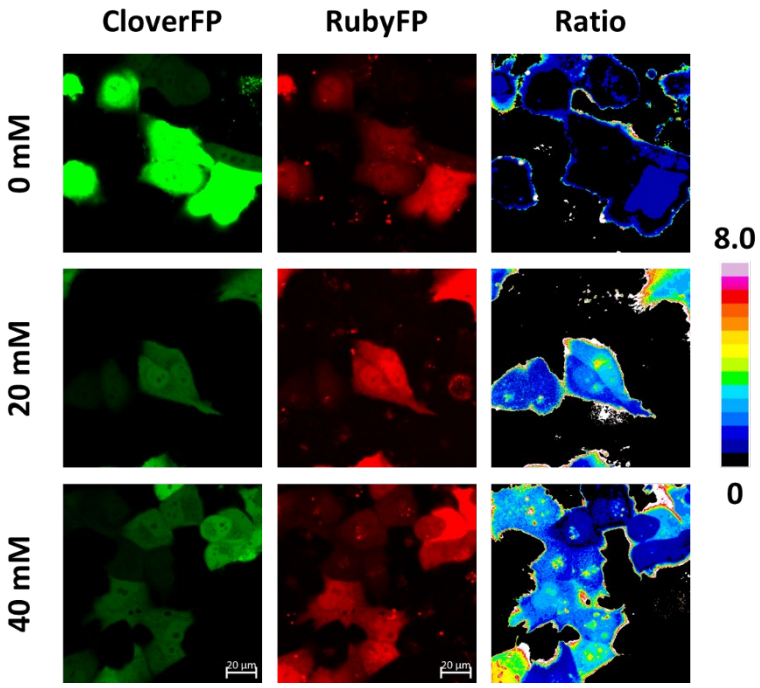


Figure S8. Ratiometric imaging of single cell after incubation with Mg^{2+} at different concentration. 0 mM, 20 mM, 40 mM of Mg^{2+} were introduced into the cell culture media. Ratiometric channel showed the RubyFP / CloverFP value from 0 to 8.0. Scale bar = 20 μm.

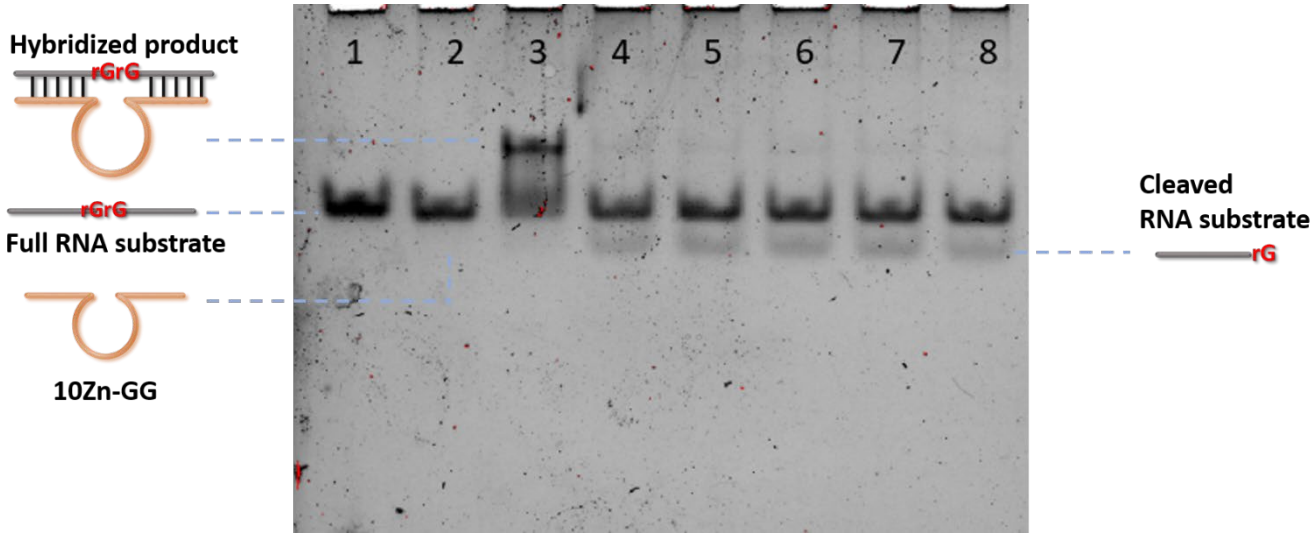


Figure S9. Gel electrophoresis analysis of the RNA cleavage efficiency using 10Zn-GG with different concentrations of Zn^{2+}. RNA substrate (lane 1), 10Zn-GG (lane 2), RNA substrate and DNAzyme in 0, 20, 50, 100, 200, 500 μM Zn^{2+} (lane 3 to lane 8).

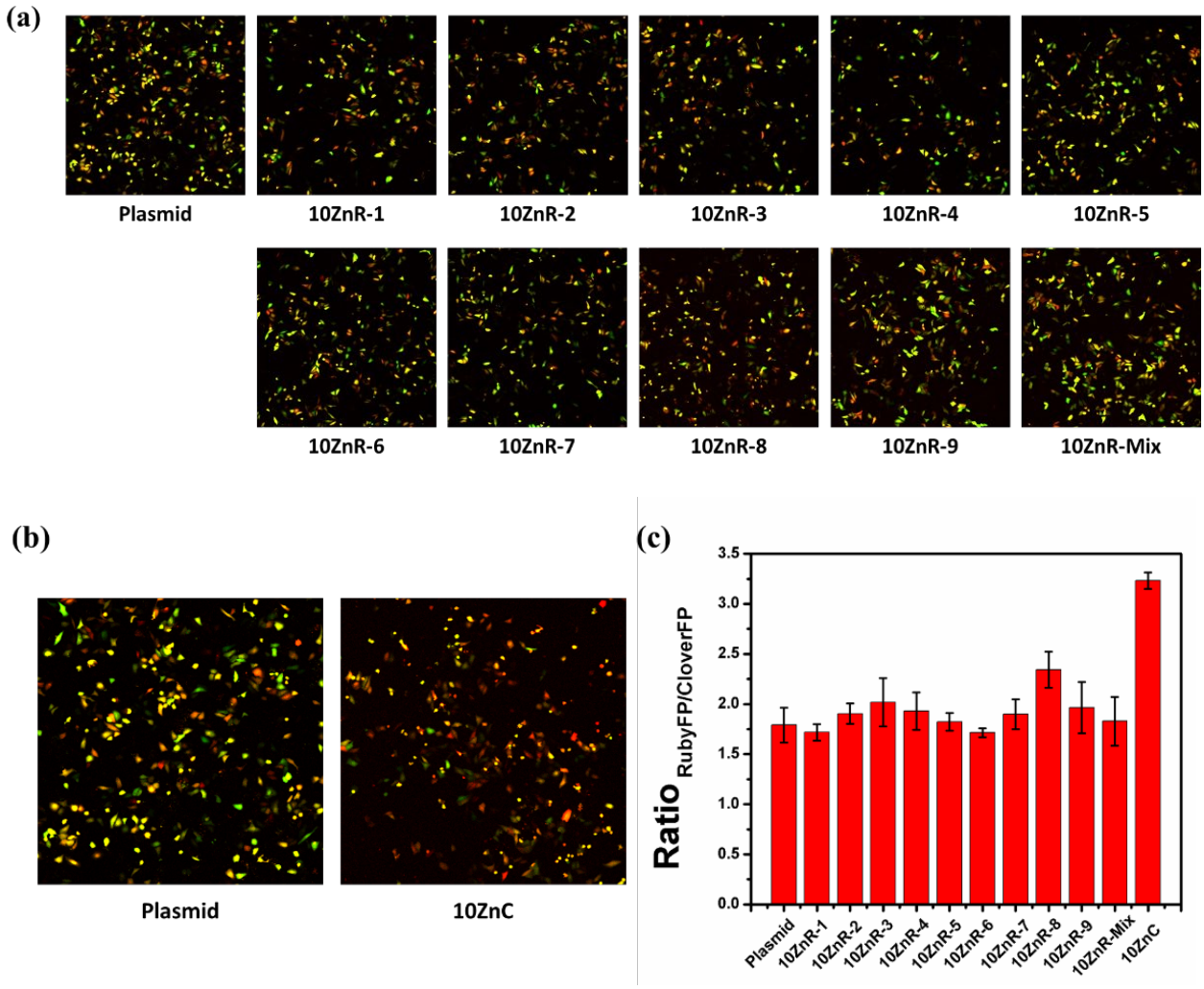


Figure S10. (a) Confocal images of cells transfected with plasmids only, plasmids and 8-17 DNAzymes toward mRNARubyFP (10ZnR-n, n=1,2,3…9), and the mixture of 10ZnR-n. (b) Confocal images of cells transfected with plasmids only and plasmids with 10ZnC. (c) Quantification result (whole ROI) of the confocal images shown in mean and S.D. While the 8-17 DNAzymes targeting $mRNA_{RubyFP}$ (10ZnR-n, n=1,2,3…9) could not suppress the expression of RubyFP, attributable to the dense structure of $mRNA_{RubyFP}$, 10ZnC targeting the $mRNA_{CloverFP}$ was able to downregulate the CloverFP under endogenous Zn^{2+}.

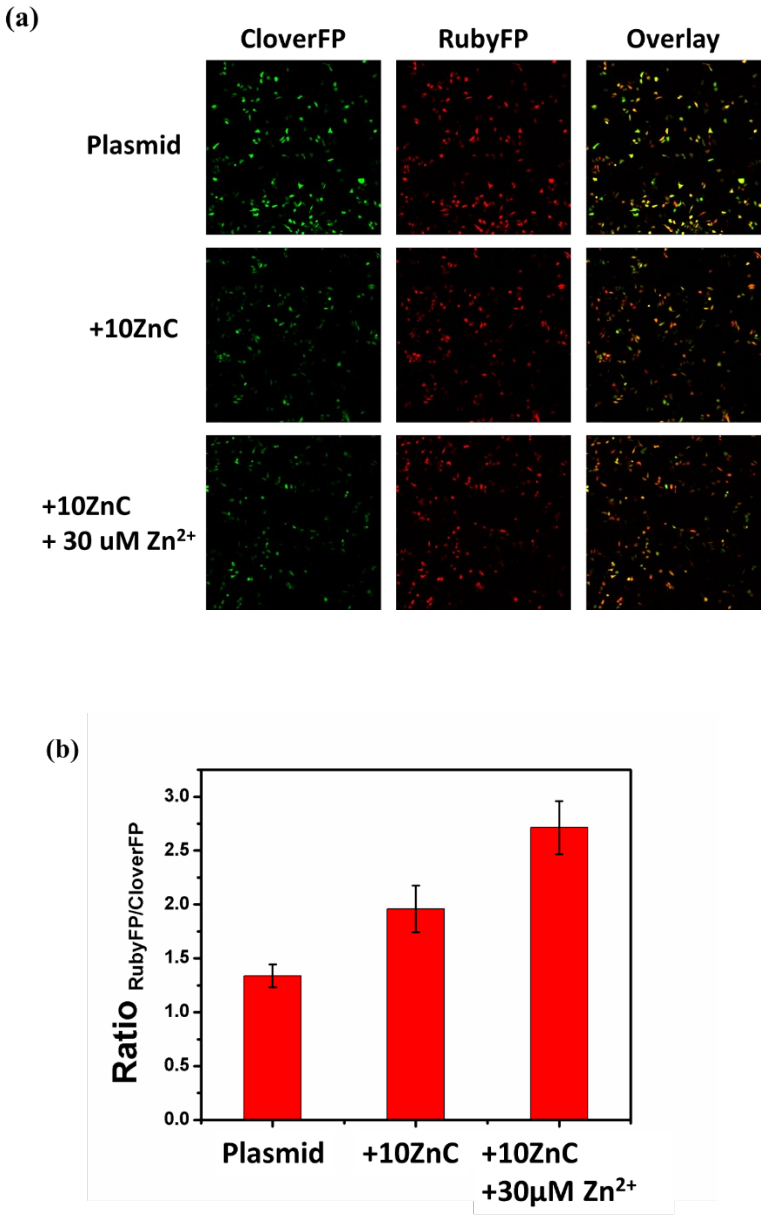


Figure S11. (a) Confocal images of cells transfected with plasmids only, plasmids with 10ZnC, and plasmids with 10ZnC and 30 μM Zn^{2+}. (b) Quantification result (whole ROI) of the confocal images from (a), shown with mean and S.D.

References

[1] a) M. Zuker, *Science* **1989**, *244*, 48-52; b) A. Abdelgany, M. Wood, D. Beeson, *J. Gene Med.* **2007**, *9*, 727-738.