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Electronic Supplementary Information (ESI)

for

DNA Octahedral Amplifier for Endogenous CircRNA Detection and Bioimaging in Living cells and its Biomarker Study

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1. Apparatus

The T20S Series Thermal Cycler for Porous Plates was purchased from Long Gene (China), the Fire-Reader V10 Gel Imager was purchased from UVITEC (UK). Fluorescence measurements were recorded using an RF-6000 spectrophotometer (Japan), and AFM characterization of the sample was observed by Bruker Multimode V8 Scanning Probe Microscopy (USA). Cell Counting Kit-8 (CCK-8) was performed at 450 nm using Thermo Fisher Scientific's Varioskan Flash microplate reader (China). Confocal fluorescence images of cells were obtained using FV3000 laser-scanning confocal microscopy (Olympus, Japan).

2. AFM imaging

10 μL of DOA nanoprobe (20 nM) was deposited on the surface of freshly cleaved mica, then the solution dried at room temperature, then the samples were observed on a Multimode V8 using Scan Asystmode.

3. Selective experiments

Various oligonucleotide targets (25 nM) including circHIPK3, miRNA21, miRNA26a, miRNA203, and miRNA1246 were added in 300 μL Tris-HCl buffer (20 mM Tris-HCl, 50 mM $MgCl_2$ and 1.0 mM EDTA, pH=7.6) containing 50 nM DOA nanoprobe, respectively. Then the mixture was kept at 37 °C for 3 h to react. The fluorescence emission spectra were recorded from 550 to 750 nm by the excitation wavelength at 540 nm. All fluorescence experiments were repeated 3 times.

4. Cell culture and CCK-8 assay

A549 and H1299 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (100×), and HEK 293T cells were cultured using DMEM medium containing 10% FBS and 1% penicillin-streptomycin solution (100×). All cells were seeded in dishes and cultured at 37 °C in a humidified atmosphere including 5% CO_2 for 24 h.

The CCK-8 assay was adopted to study the cytotoxicity of the DOA nanoprobe. In details, A549 cells (8×10^3 cells/well) were cultivated in 96-well plates in a humidified

atmosphere with 5% CO_2 at 37 °C. After incubating for 24 h, the A549 cells were treated with DOA nanoprobe (0, 100, 150 nM) for 6, 12 and 24 h, respectively. Ultimately, the cells were washed with 1× PBS for three times and 90 μL of new complete medium and 10 μL of DMSO dissolved CCK-8 were added into each well, and incubated for 35 min. Cytotoxicity data were obtained by measuring the absorbance value (450 nm) and untreated cells were used as the negative controls.

5. Flow cytometry assay

A549 cells were planted in Petri dishes and cultured in a humidified atmosphere with 5% CO_2 at 37 °C. After treatment with DOA nanoprobe (150 nM) for 6 h, the cells were detached from culture dishes using trypsin solution. Then the treated cells were centrifuged at 1000 rpm for 4 min and resuspended in PBS solution for three times. Flow Cytometry Assay was performed by BD FACSCanto Flow Cytometer (USA) using fluorescent channel Cy5 (633 nm excitation).

6. Real-time quantitative PCR (RT-qPCR)

Total RNAs were extracted from cells using Trizol reagent (Sangon Biotech Engineering Technology & Services, China) according to the manufacturer's instructions. The concentration of total RNA was detected using an ultraviolet spectrophotometer. 1.0 μg of total RNA was used for cDNA synthesis by Hifair II 1st Strand cDNA Synthesis Super Mix for qPCR (gDNA digester plus) kit. Then qPCR was conducted by using a SYBR Green PCR Kit, and samples were run in triplicate by Roche (USA) RT-qPCR machine. The GAPDH primers were used as an internal control for RT-qPCR, and the results were analyzed using the $2^{-\Delta\Delta CT}$ method and normalized to the GAPDH transcript. The primers used for RT-qPCR in this study are shown in Table S1.

7. Supplementary figures and tables

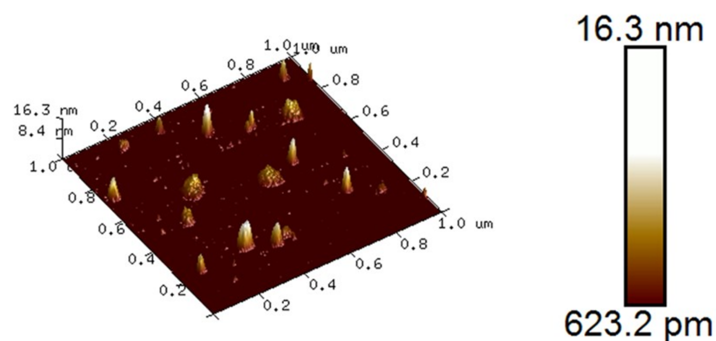

Figure S1. Characterization of DOA nanoprobe by atomic force microscopy (AFM).

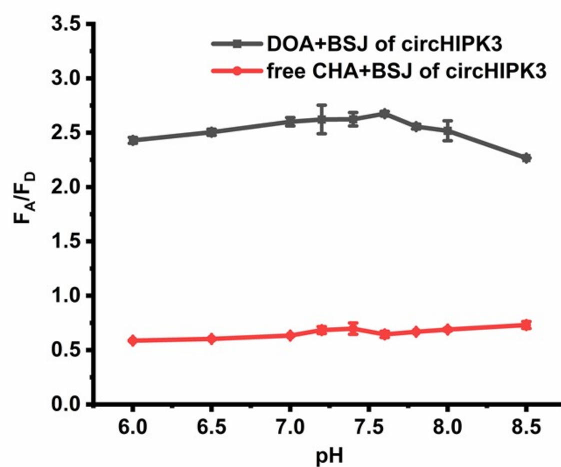

Figure S2. The corresponding fluorescence intensity ratio of signal (F_A/F_D) values of DOA nanoprobe (50 nM) in Tris-HCl buffer of different pH, the mixture is incubated for 3 h at 37 °C. Mean ± SD, n = 3.

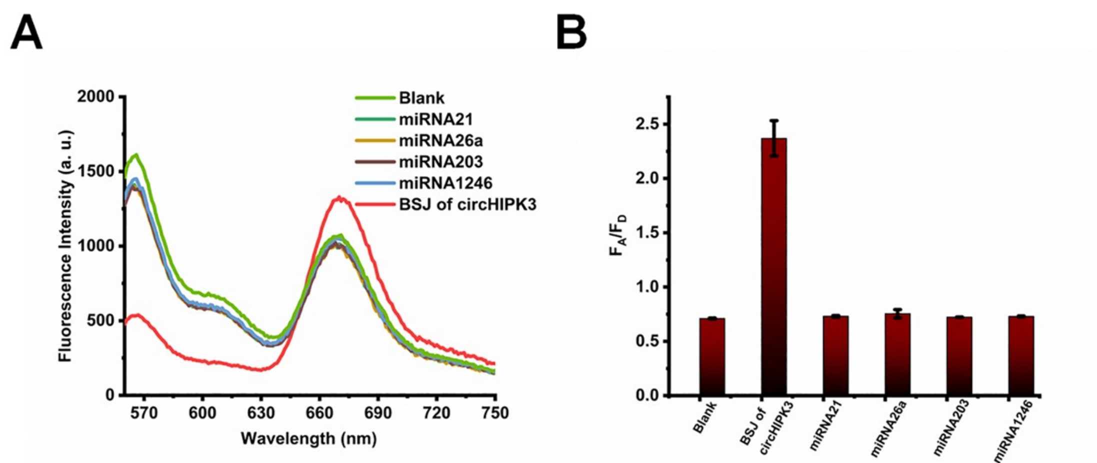

Figure S3. (A) Fluorescence spectra and (B) histogram of the corresponding fluorescence intensity ratio (F_A/F_D) of 50 nM DOA nanoprobe towards different RNAs in Tris-HCl buffer (20 mM Tris-HCl, 50 mM $MgCl_2$ and 1.0 mM EDTA, pH=7.6). The concentration of DOA nanoprobe and RNAs are 50 nM and 25 nM. the mixture is incubated for 3 h at 37 °C, respectively. Mean ± SD, n = 3.

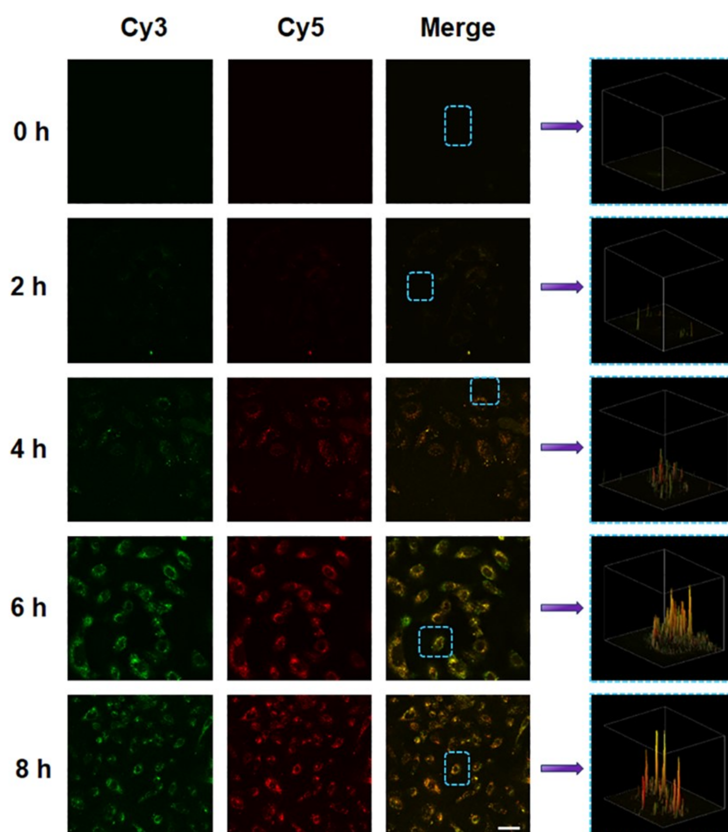

Figure S4. The optimization of incubation time for DOA nanoprobe in living cells. A549 cells are incubated with 150 nM DOA nanoprobe for different time at 37 °C for confocal microscopy imaging, scale bar is 30 μm.

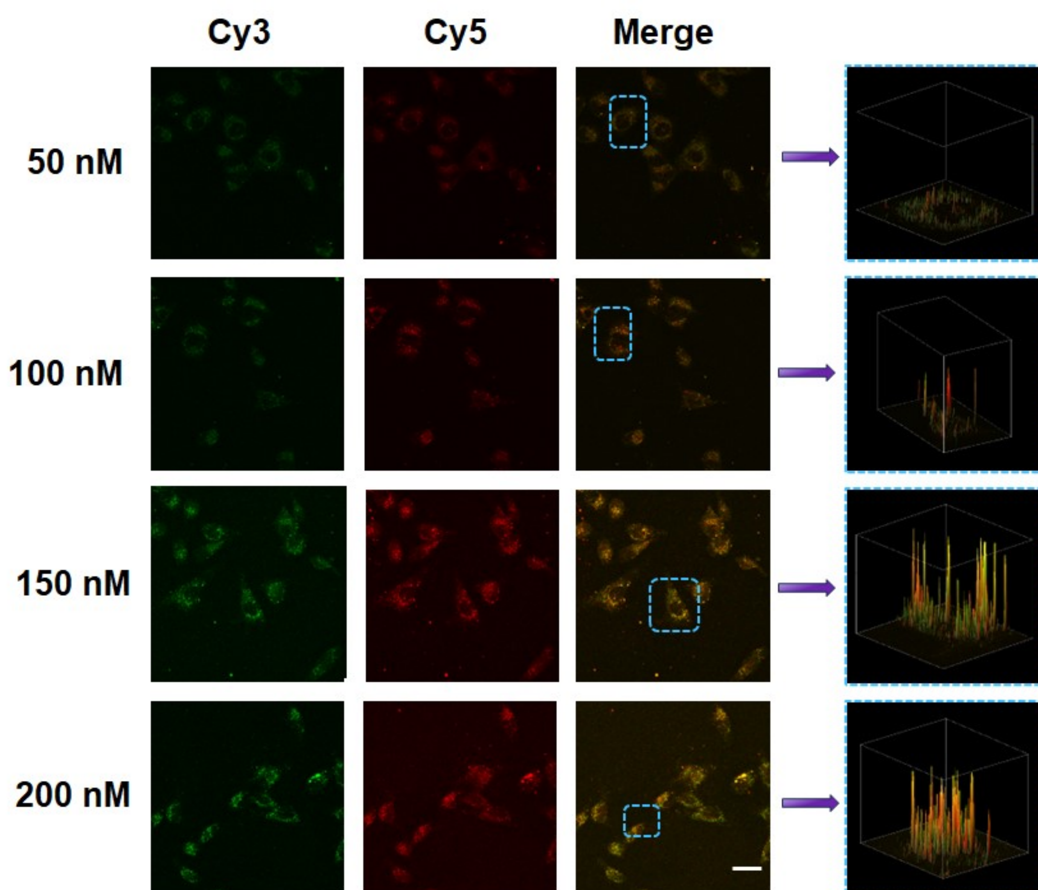

Figure S5. The optimization of concentration for DOA nanoprobe in living cells. A549 cells are incubated with DOA nanoprobe for different concentration at 37 ℃ for confocal microscopy imaging, scale bar is 30 μm.

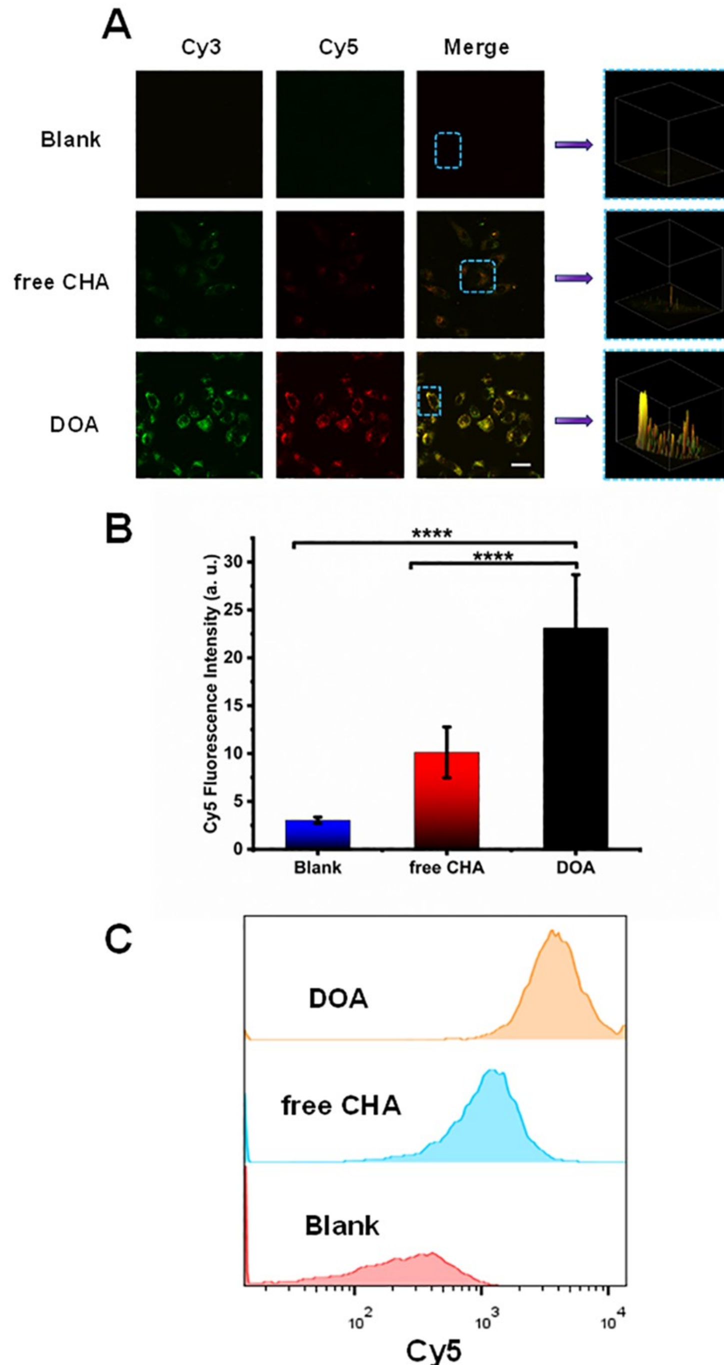

Figure S6 (A) Confocal fluorescence images of circHIPK3 in A549 cells incubated with DOA nanoprobe (150 nm) and free CHA (150 nm) for 6 h at 37 °C, respectively. Scale bar is 30 μm. (B) Histogram of the Cy5 fluorophore fluorescence intensity of signal values. Numerical values in the graph indicate the mean value ± SD from 20 cells. *t*-Test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (C) Flow cytometry assay, A549 cells are incubated with the DOA nanoprobe (150 nM) and free CHA (150 nM) for 6 h at 37 °C, respectively.

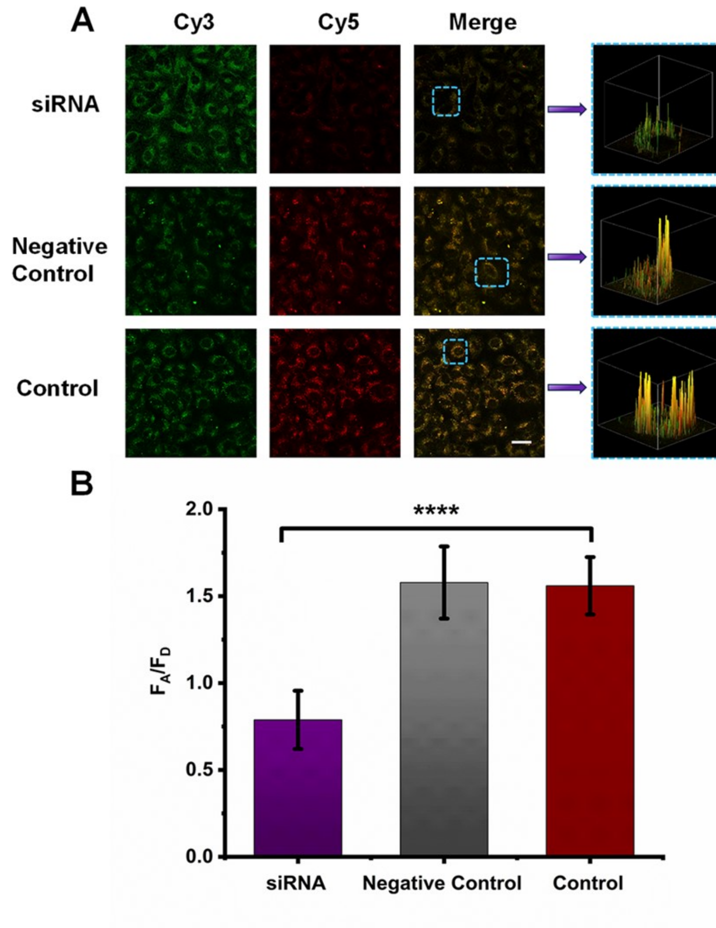

Figure S7. (A) Confocal fluorescence images of A549 cells are treated with the siRNA, scramble (Negative Control) and untreated (control) by 150 nM DOA nanoprobe. Scale bar is 30 μm. (B) Histogram of the corresponding fluorescence intensity ratio of signal (F_A/F_D) values. Numerical values in the graph indicate the mean value ± SD from 20 cells. *t*-Test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Table S1. The sequences of DNA and RNA used in this study

	The sequence of DNA and RNA (from left to right: 5’ to 3’)
S-1	ATCACCCAAACCCTCAATCTGCGAGTGTCTTTGG CATACTTCACCAGGTTGAATCCTATGCTCGTACA TTGTCGCAGTTCAGATACGCTTCATACTGAGAGC GTTCCG
S-2	GGATACATTATACGGTGGTTTGTACGAGCATAG GATTCTTCCTGGTGAAGTATGCCAATTGGATCCT AAATTCTTGCG
S-3	ATCACCCAAACCCTCAATCTCGAAAGTGATCCA CCGTATAATGTATCCTTCGAGCAGCACGAACTG TCTTCATTCGTCGTCGTCGTAGTTCTGTAGCCTC TTATGCGGA
S-4	GCGTATCTGAACTGCGACTTCCGCATAAGAGGC TACAGTTGGACCGTAGTTAAATGACTTCGGAAC GCTCTCAGTATGTTTATCAATCATACGAACCGAC T
S-5	CGGTCATCGTCGTCGTCGTTGCACGAATACGAA TACTATTCGCAAGAATTTAGGATCCTTGTCATTT AACTACGGTCC
S-6	CGACGACGACGATGACCGTTCTACGACGACGAC

	GAATGTTGACAGTTCGTGCTGCTCGTTTAGTATT CGTATTCGTGCTTTATCAATCATACGAACCGACT
H1	AGATTGAGGGTTTGGGTGATTTTTGAGGCCATA CCTGTAGTACCGACCA/iCy3-dT/GTGTAGATCGGTACTACAGGTA
H2	AGTACCGATCTACACA/iCy5-dT/GGTCGGTACTACAGGTACCATGTGTAGATTT AGTCGGTTCGTATGATTGAT
circHIPK3	**GUAUGGCCUCA**CAAGUCUUGGUCUACCCACCA UAUGUUUAUCAAACUCAGUCAAGUGCCUUUUG UAGUGUGAAGAAACUCAAAGUAGAGCCAAGCA GUUGUGUAUUCCAGGAAAGAAACUAUCCACGG ACCUAUGUGAAUGGUAGAAACUUUGGAAAUUC UCAUCCUCCCACUAAGGGUAGUGCUUUUCAGA CAAAGAUACCAUUUAAUAGACCUCGAGGACAC AACUUUUCAUUGCAGACAAGUGCUGUUGUUUU GAAAAACACUGCAGGUGCUACAAAGGUCAUAG CAGCUCAGGCACAGCAAGCUCACGUGCAGGCA CCUCAGAUUGGGGCGUGGCGAAACAGAUUGCA UUUCCUAGAAGGCCCCCAGCGAUGUGGAUUGA AGCGCAAGAGUGAGGAGUUGGAUAAUCAUAGC AGCGCAAUGCAGAUUGUCGAUGAAUUGUCCAU

	ACUUCCUGCAAUGUUGCAAACCAACAUGGGAA AUCCAGUGACAGUUGUGACAGCUACCACAGGA UCAAAACAGAAUUGUACCACUGGAGAAGGUGA CUAUCAGUUAGUACAGCAUGAAGUCUUAUGCU CCAUGAAAAAUACUUACGAAGUCCUUGAUUUU CUUGGUCGAGGCACGUUUGGCCAGGUAGUUAA AUGCUGGAAAAGAGGGACAAAUGAAAUUGUAG CAAUCAAAAUUUUGAAGAAUCAUCCUUCUUAU GCCCGUCAAGGUCAAAUAGAAGUGAGCAUAUU AGCAAGGCUCAGUACUGAAAAUGCUGAUGAAU AUAACUUUGUACGAGCUUAUGAAUGCUUUCAG CACCGUAACCAUACUUGUUUAGUCUUUGAGAU GCUGGAACAAAACUUGUAUGACUUUCUGAAAC AAAAUAAAUUUAGUCCCCUGCCACUAAAAGUG AUUCGGCCCAUUCUUCAACAAGUGGCCACUGC ACUGAAAAAAUUGAAAAGUCUUGGUUUAAUUC AUGCUGAUCUCAAGCCAGAGAAUAUUAUGUUG GUGGAUCCUGUUCGGCAGCCUUACAGGGUUAA AGUAAUAGACUUUGGGUCGGCCAGUCAUGUAU CAAAGACUGUUUGUUCAACAUAUCUACAAUC**U** **CGGUACUACAG**
BSJ of circHIPK3	TCGGTACTACAGGTATGGCCTCA

siRNA	**Sense**-GGUACUACAGGUAUGGCCUTT **Antisense**-AGGCCAUACCUGUAGUACCTT
circHIPK3 for human	**Forward**-TATGTTGGTGGATCCTGTTCGGC **Reverse**-TGGTGGGTAGACCAAGACTTGTGA
GAPDH mRNA for human	**Forward**-CCAAGGTCATCCATGACAAC **Reverse**-GCTTCACCACCTTCTTGATG

*Red and bold mark the recognition sequence of circRNA

Table S2. Comparison of DOA nanoprobe with other methods

Method	Advantages of the DOA nanoprobe compared with this method	Reference
PCR method	The DOA nanoprobe enables *in situ* monitoring intracellular circRNA, offers eliminates temperature cycling and no complex operation, but PCR method detect RNA in cell lysates and can't reflect the real-time RNA expression levels in living cells.	[1]
Northern blotting method	The DOA nanoprobe enables *in situ* detection and bioimaging in living cells, and provides the spatial-temporal information, but the northern blotting	[2]

	method can only be performed in cell lysate, and the northern blotting shows the low sensitivity, requires large amounts of RNA sample.	
RNA sequencing (RNA-seq)	The DOA nanoprobe enables *in situ* detection and bioimaging in living cells, and offers the simple operation, but the RNA-seq has the relatively high costs, time consuming and complicated analysis of data.	[3]
Fluorescence in situ hybridization (FISH)	FISH is only performed in fixed cells or tissues by fluorescently labeled DNA probes for hybridization, and it can't provide the spatial-temporal information. While the DOA nanoprobe has better permeability for *in situ* detection and bioimaging.	[4]
Traditional fluorescence nanoprobes	The traditional fluorescence nanoprobes cannot accurately detection the low abundance circRNA expression levels in living cells, and it generates low detection signal. While the DOA nanoprobe can	[5]

	accurately detect low expression level circRNA in living cells by enzyme-free signal amplification.	
Conventional enzyme-free amplification method	Some enzyme-free amplification methods, such as CHA, RCA and HCR, usually depend on transfection system to deliver DNA hairpin probes into cells, it has poor biocompatibility in cell biology, and they were not possible with rigorous equal amount, which would able to affect amplification efficiency. However, the DOA nanoprobe has excellent capability of self-delivery without transfection agents, and enables significantly increase the efficiency of amplification reaction.	[6]

8. References

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