Supplementary Information (SI)

for

Sensitive detection and accurate bioimaging of circRNA based on sponge amplification using a DNA tetrahedral nanoprobe

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1. Reagents and materials

All DNA and RNA oligonucleotides were procured from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China), and the sequences of all DNA and RNA are listed in Table S1. The fluorescence measurements were recorded using an RF-6000 spectrophotometer (Shimadzu, Japan), while the AFM measurement was conducted with a Bruker Multimode V8 scanning probe microscope (USA). The confocal fluorescence images of cells were obtained using an Olympus FV3000 laser scanning confocal microscope (Japan). The 20S series porous plate thermal cycler was purchased from Long Gene Company (China), while the Fire-Reader V10 gel imaging system was acquired from UVITEC Ltd. (United Kingdom). The corning Roswell Park Memorial Institute Medium 1640 (RPMI 1640), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were provided by Coring (USA). The Hifair II 1st Strand cDNA Synthesis Super Mix (gDNA digester plus) and the Hieff qPCR SYBR Green Master Mix were provided by Yeasen Biotech (China). The cell Kit-8 (CCK-8) and Tris-HCl were provided by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The CCK-8 was utilized for cell quantification by the Varioskan Flash microplate reader from Thermo Fisher Scientific (China) at 450 nm. The human non-small cell lung cancer cell lines (A549), human liver cancer cell line (HepG2), human normal liver (LO2) and cervical cancer cells (HeLa) were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Ultra-pure water (18 MΩ/cm) was obtained from the Milli-Q Reference System (Billerica, MA, USA). The tissue samples were obtained from Ruijin Hospital in Shanghai. All experimental protocols have been approved by the Ethics Committee of the Medical College of Ningbo University.

2. Agarose gel electrophoresis

The assembled DTNP was first verified using agarose gel electrophoresis. 10 μL of DNA samples and 2 μL of 6×loading buffer were mixed thoroughly. The mixture was sequentially added to each well, and the gel was run at 90 V for approximately 45 min under low-temperature conditions in 1×TBE buffer (Tris-borate-EDTA, pH 8.3). Finally, the voltage operation was ceased and the gel was removed carefully, then the Fire Reader V10 was used to capture images of the gel.

3. Atomic force microscopy (AFM)

To prepare the sample, 10 nM DTNP solution (10 μL) was slowly added onto the freshly cleaved mica surface using a pipette. The sample was then dried at room temperature and ultimately observed using the ScanAsyst mode in Multimode V8.

4. Transmission electron microscopy (TEM)

10 μL of DTNP solution was added onto the surface of the copper mesh. Subsequently, the sample was heated for 2 min at infrared radiation. After the sample was completely dried, the sample was characterized using JEM-2100 transmission electron microscope.

5. Cell culture

The LO2 cells and A549 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin solution, 1 mM sodium pyruvate solution and 2.5 g/L D-glucose solution. The HepG2 cells and HeLa cells were maintained in DMEM medium containing 10% FBS and 1% penicillin-streptomycin solution. All cells were inoculated into culture dishes and incubated in a humidified environment at 37 °C with 5% CO_2 for a certain time.

6. Cytotoxicity assay

The cytotoxicity of DTNP in cells was investigated using the CCK-8 Assay. Hela cells were first seeded into a 96-well plate (5000 cells per well) and incubated in an incubator at 37 ℃ with 5% CO_2. After incubating for 24 h, Hela cells were treated with DTNP at concentrations of 0 nM, 25 nM, 50 nM and 100 nM, and then incubated for 12 h and 24 h. Next, the cells were washed three times with PBS, 90 μL of fresh culture medium and 10 μL of CCK-8 were added into each well, then the cells were incubated for 60 min until a change of noticeable color. The absorbance value at 450 nm was ultimately measured using a microplate reader to obtain cytotoxicity data.

7. The extraction of total RNA from cells and tissues

Firstly, cells were washed three times with PBS after removing the culture medium, then 1 mL Trizol was added into cells for 5 min to cell lysis. Secondly, 200 μL of chloroform was added into cell lysate in an EP tube, the mixture was mixed thoroughly by vortexing, then sited for 5 min. After centrifugation at 12000 rpm for 10 min at 4 °C, the supernatant was transferred carefully to a new RNase-free EP tube and added 500 μL of isopropanol, vortexed to uniformly, then sited for 5 min. Thirdly, the supernatant was discarded once more by centrifuging at 12,000 rpm for 10 min at 4 °C, and the RNA pellet was washed with 1 mL of 75% ethanol (prepared with DEPC water) by centrifuging at 7500 rpm for 5 min at 4 °C. The washed RNA pellet should be placed in ambient air to dry completely. For total RNA extraction from tissue samples, the tissue sample was first cut into a small piece of frozen tissue sample by a low-temperature mortar, and promptly weighed it. Then tissue sample was transferred immediately into an RNase-free centrifuge tube added 1 mL Trizol. Subsequent steps should proceed according to those described above for total RNA extraction from cells.

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

Total RNA was extracted from cells using Trizol reagent (China National Biotechnology Group Corporation). The total RNA concentration was measured using the NanoDrop One (Thermo Fisher Scientific). According to the manufacturer's instructions from a Hifair II 1st Strand cDNA Synthesis Super Mix for qPCR (gDNA digester plus) kit, 1 μg of total RNA was utilized for cDNA synthesis. Then, according to the manufacturer's instructions from a SYBR Green PCR Kit, the reagent and cDNA were mixed for qPCR. Subsequently, the mixtures were run in triplicate by Roche 96 (USA) RT-qPCR machine. Finally, the results were analyzed using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH transcript levels.

9. Supplementary figures and table

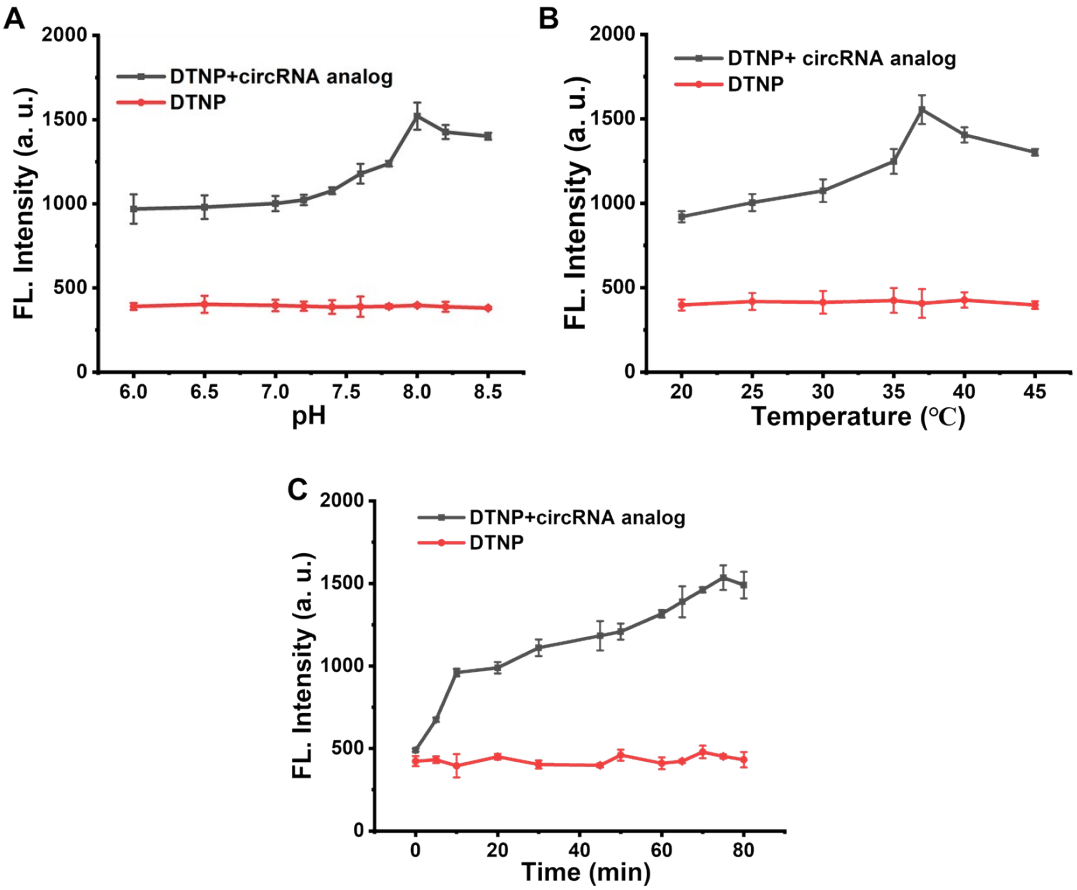

Fig. S1 (A) Fluorescence intensity of DTNP with/without 90 nM circRNA analog in different pH buffer solution. (B) Fluorescence intensity of 30 nM DTNP with/without 90 nM circRNA analog under different temperatures. (C) Fluorescence intensity of 30 nM DTNP with/without 90 nM circRNA analog at different time. Tris-HCl buffer solution: 20 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0. λ_{ex} = 540 nm, λ_{em} = 550~650 nm.

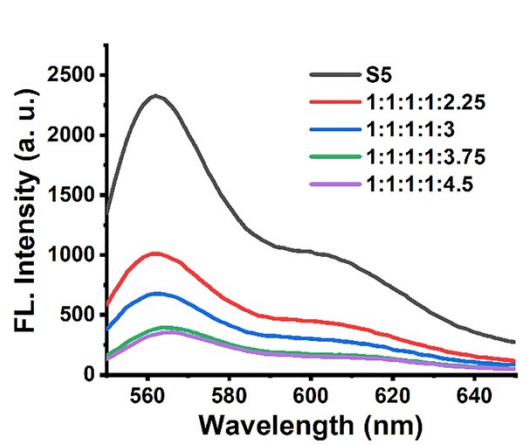

Fig. S2 Fluorescence spectra of S1, S2, S3, S4, and S5 assemblies synthesized with hybridization ratios ranging from 1:1:1:1:2.25 to 1:1:1:1:4.5 in Tris-HCl buffer solution (20 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0). λ_{ex} = 540 nm, λ_{em} = 550~650 nm.

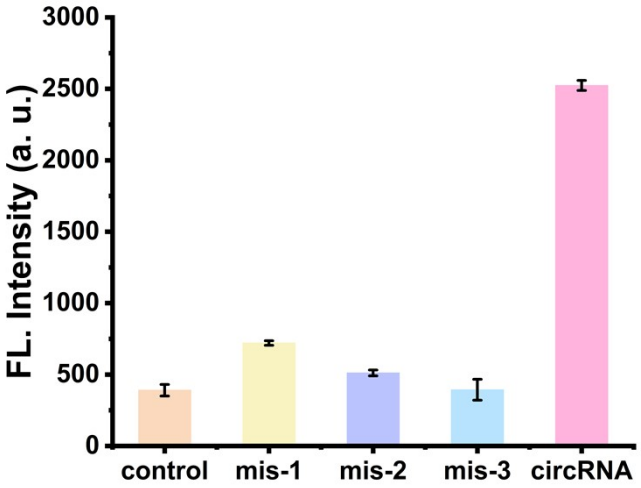

Fig. S3 The fluorescence intensity of DTNP (30 nM) measured with the circRNA analog (90 nM), single-base mismatched (mis-1), double-base mismatched (mis-2), and triple-base mismatched analogs (mis-3), using DTNP without the circRNA analog as a control. Mean ± SD, n = 3. Tris-HCl buffer solution: 20 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0. λ_{ex} = 540 nm, λ_{em} = 550~650 nm.

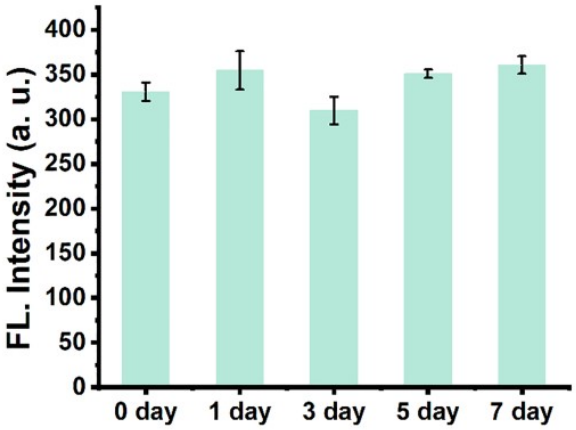

Fig. S4 The fluorescence intensity of DTNP (30 nM) in Tris-HCl buffer solution (20 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0) for standing 7 days. λ_{ex} = 540 nm, λ_{em} = 550~650 nm.

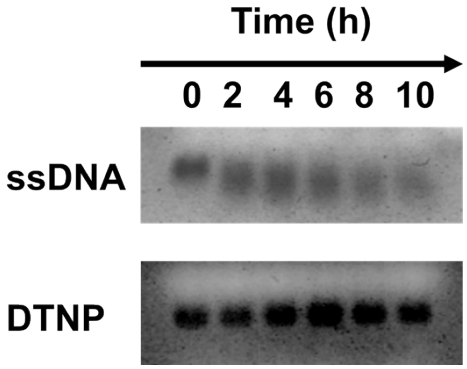

Fig. S5 The degradation of DTNP and single-stranded DNA (ssDNA) in the presence of 10% FBS at 37 °C using agarose gel electrophoresis.

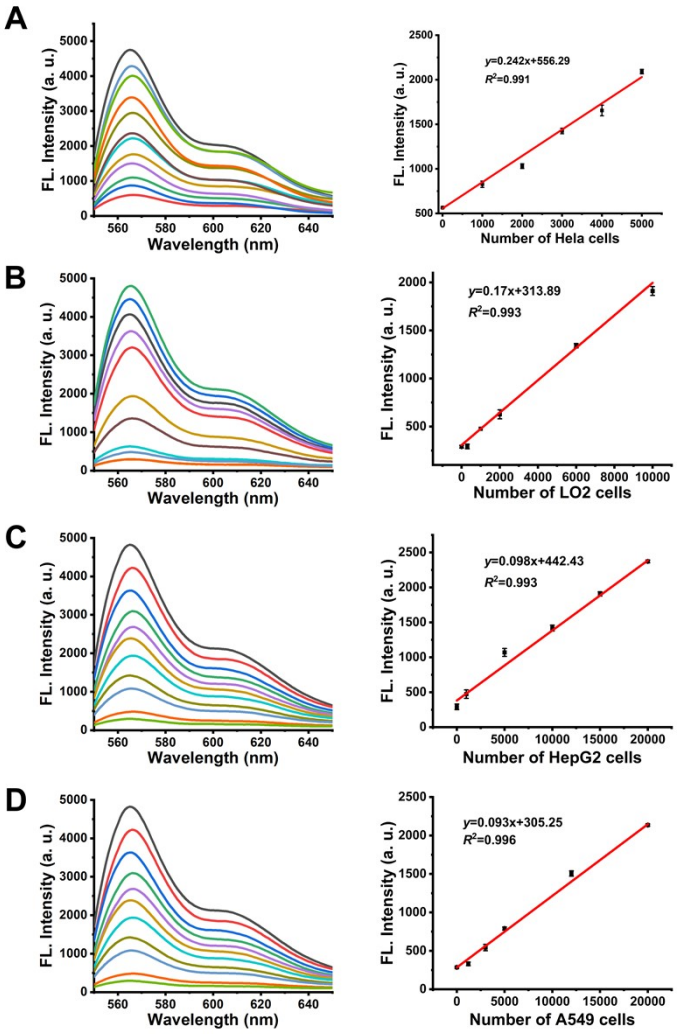

Fig. S6 (A) Fluorescence spectra of DTNP in number of HeLa cells (0 ~ 1×10^5 cells, left) and linear relationship between fluorescence intensity and the number of HeLa cells (1×10^2 ~ 5×10^3 cells, right); (B) Fluorescence spectra of DTNP in number of LO2 cells (0 ~ 1.3×10^5 cells, left) and linear relationship between fluorescence intensity and the number of LO2 cells (3×10^2 ~1×10^4 cells right); (C) Fluorescence spectra of DTNP in number of HepG2 cells (0 ~ 1.8×10^5 cells, left) and linear relationship between fluorescence intensity and the number of HepG2 cells (0 ~ 2×10^5 cells, right); (D) Fluorescence spectra of DTNP in number of A549 cells (0 ~ 2×10^5 cells, left) and linear relationship between fluorescence intensity and the number of A549 cells (0 ~ 2×10^4 cells, right). Mean ± SD, n = 3. Tris-HCl buffer solution: 20 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0. λ_{ex} = 540 nm, λ_{em} = 550~650 nm.

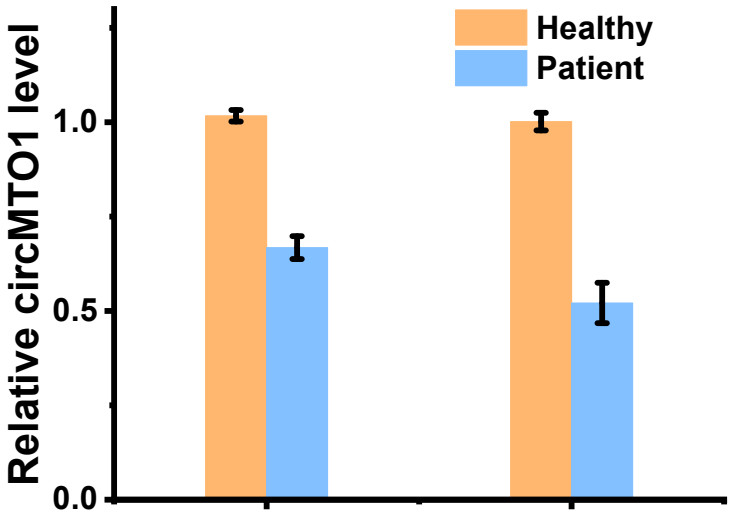

Fig. S7 Analysis of circMTO1 expression in tissues by RT-qPCR (500 ng total RNA per replicate). Mean ± SD, n = 3. Tris-HCl buffer solution: 20 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0. λ_{ex} = 540 nm, λ_{em} = 550~650 nm.

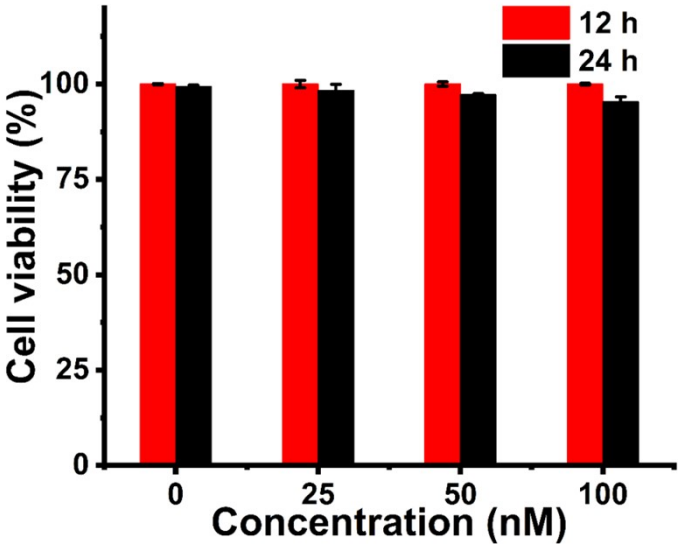

Fig. S8 The cell viability of HeLa cells with different concentrations of DTNP (0 nM, 25 nM, 50 nM, and 100 nM) at 12 h and 24 h, respectively.

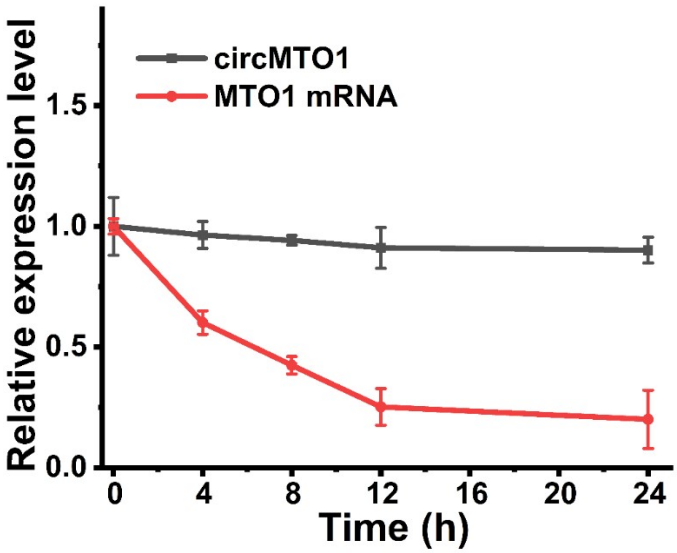

Fig. S9 CircMTO1 and MTO1 mRNA expression levels in HeLa cells by RT-qPCR after treatment with transcriptional inhibitor actinomycin D (5 μg/mL) across varying time periods.

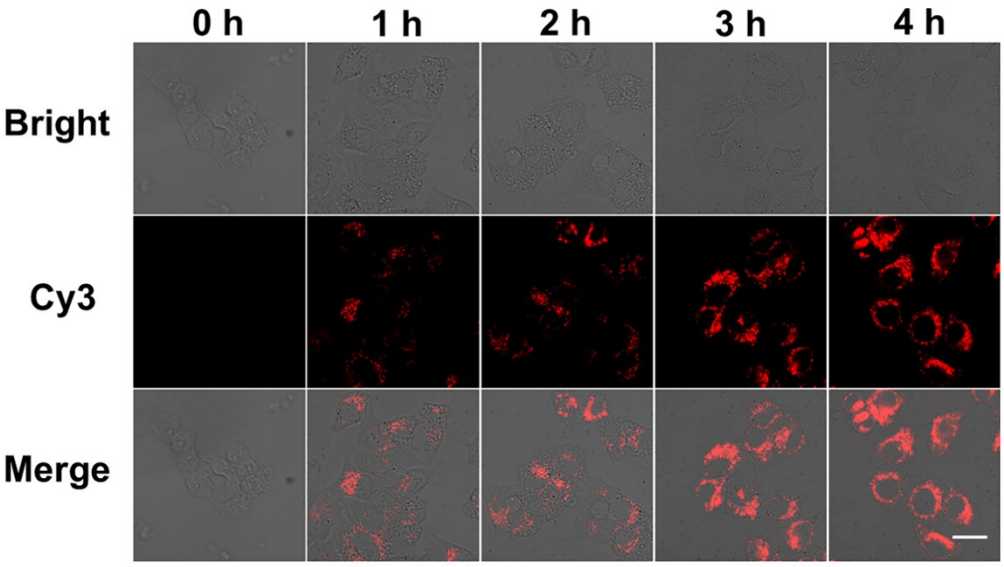

Fig. S10 Confocal fluorescence imaging of HeLa cells with 50 nM DTNP at different time (0 h, 1 h, 2 h, 3 h, 4 h), scale bar: 30 μm.

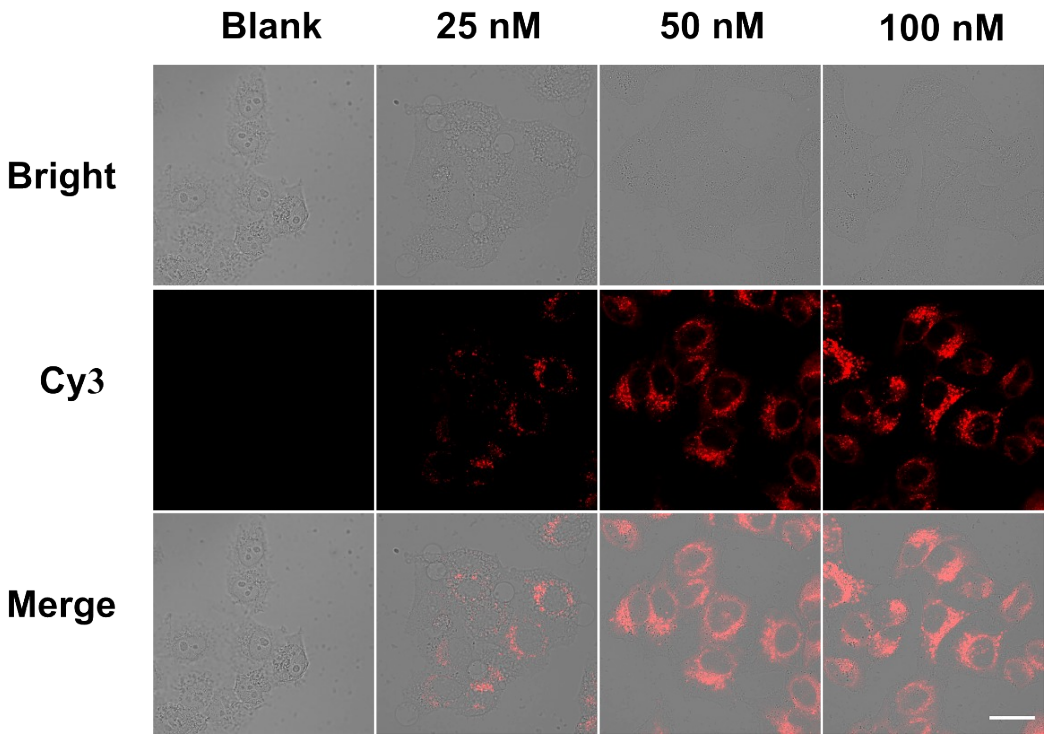

Fig. S11 Confocal fluorescence imaging of HeLa cells with the different concentration of DTNP (0, 25, 50, 100 nM), scale bar: 30 μm.

Table S1. The sequence of DNA and RNA information.

Name	Sequence (5'-3')
S1	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGC CGCCATAGTATTTTTTTTTCAGAGCCG-BHQ2
S2	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAG GGTCCAATACTTTTTTTTTTCAGAGCCG-BHQ2
S3	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATG GCGGCTCTTCTTTTTTTTTTCAGAGCCG-BHQ2
S4	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGA CCCTCGCAT
S5	Cy3-CGGCTCTGGGTCTGTGGGGA
circMTO1	GUCAGAUGUCAUGUAAUCCUUCCUUUGGUGGCAUCGGAAAGGG ACAUUUAAUGAGGGAAGUAGAUGCCUUGGAUGGCCUGUGUUCU CGCAUCUGUGACCAGUCUGGUGUACAUUAUAAAGUAUUAAACC GGCGUAAGGGACCAGCUGUGUGGGGUCUGAGAGCUCAGAUUGA UAGGAAACUCUAUAAACAGAACAUGCAGAAAGAAAUCUUGAAU ACACCACUGCUUACUGUUCAGGAGGGAGCUGUAGAAGAUCUUA UUCUUACAGAACCAGAGCCUGAACACACUGGGAAAUGCCGUGU CAGUGGGGUUGUUUUGG
linear RNA	P-AAGAUCUUAUUCUUACAGAACCAGAGCCU
adsorption site	AUCUUAUUCUUACAGAACCAGAG
mis-1	ATCTTATTCTTACAGAAC*T*AGAG
mis-2	ATCTTATTCTTAC*T*GAAC*T*AGAG

mis-3	ATCTTATTCTTAC*T*GAAC*TG*GAG
circMTO1-F	GAGCTGTAGAAGATCTTATTC
circMTO1-R	CACAGGCCATCCAAGGCATC
MTO1 F	TGCATCAGAGGCTTGGAGAA
MTO1 R	AAGGAAGGGGTGATCTGACG
GAPDH-F	ATCTCTGCCCCCTCTGCTGA
GAPDH-R	GATGACCTTGCCCACAGCCT

Table S2. Comparison of DTNP with other methods

Method	Time	Biological sample	Advantages of DTNP compared with this method	Reference
HCR	90 min	Cells	The DTNP can autonomously enter cells, whereas HCR hairpin probes require transfection agents for cellular delivery	1
Ligation-based PCR	2 h	Cells	The DTNP enables direct, enzyme-free detection of circular RNAs, thereby completely avoiding the biases introduced by ligation and PCR amplification steps	2
Nanopore sequencing combined with CIRI-long	4 h	Mouse brain	Although Nanopore sequencing combined with CIRI-long can provide full-length sequence and isoform information, it requires costly equipment and involves complex data analysis, whereas DTNP offers faster detection at a lower cost	3
linear DNA nanostructure	100 min	Cells	The DTNP exhibits superior biostability and enhanced cellular uptake efficiency compared to linear DNA nanostructures, owing to its enclosed three-dimensional architecture that provides greater resistance to nuclease degradation.	4
DTNP	75 min	Tissues and cells	The DTNP is characterized by its short detection time and capability for *in situ* cellular imaging.	This work

Reference:

1. J. Jiao, G. Wang, F. Zou, S. Tang, S. Chen, P. Miao, J. W. Jiao, *Sensors and Actuators B: Chemical*. 2023, **378**, 0925-4005.

2. P. Zhang, N. Guo, K. Gao, F. Su, F. Wang and Z. Li, *Organic & Biomolecular Chemistry*, 2020, **18**, 3269-3273.
3. J. Zhang, L. Hou, Z. Zuo, P. Ji, X. Zhang, Y. Xue and F. Zhao, *Nature Biotechnology*, 2021, **39**, 836-845.
4. J. Jiao, Y. Xiang, C. Duan, Y. Liu, C. Li and G. Li, *Analytical Chemistry*, 2020, **92**, 12394-12399.