Electronic Supporting Information for:

Spherical Nucleic Acid-based Two-Photon Nanoprobe for RNase H Activity Assay in Living Cells and Tissues

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Experimental section

Materials

Trisodium citrate and chloroauric acid ($HAuCl_4 \cdot 4H_2O$) were obtained from Sigma–Aldrich (USA). Ribonuclease H (RNase H), deoxyribonuclease I (DNase I), and Ribonuclease A (RNase A) were purchased from Takara Biotechnology Co., Ltd. (Takara, China). Ribonuclease HII (RNase HII) was purchased from New England Biolabs (NEB). SYBR gold was purchased from Invitrogen (U.S.A.). Ethyl-4-[3,6-bis(1-methyl-4-vinylpyridium iodine)-9H-carbazol-9-yl]] (EBMVC) was prepared using a method described in our prior work.[1] Human RNase H and rat RNase H ELISA kits were purchased from Mlbio (China). Water used in all experiments was purified via a Milli-Q water system (Millipore, USA). All cells were provided by the Biomedical Engineering Center of Hunan University (China). Cell culture products were purchased from Clontech (Clontech, CA). Rat models were provided by the Hunan Provincial Tumor Hospital of the Central South University (China). The study was approved by the Ethics Committee of Hunan Provincial Tumor Hospital. All oligonucleotides (Table S1, Electronic Supplementary Information (ESI)) were synthesized and HPLC-purified by Sangon Biotechnology Co., Ltd. (Sangon, China).

Characterization and Instrumentation

UV-Vis absorption spectra were obtained using a Hitachi U-4100 UV/Vis spectrophotometer (Japan) in quartz cuvettes with 1 cm path length. Single-photon fluorescence was performed on a PTI QM4 Fluorescence System (Photo Technology International, Birmingham, NJ). Dynamic light scattering size measurements were performed on a Malvern Zetasizer 3000HS (UK). Two-photon microscopy of cells and tissues was performed with the emission channel of 520-580 nm upon excitation at 800 nm using an Olympus FV1000-MPE multiphoton laser scanning confocal

microscope (Japan) and a Nikon A1R MP multiphoton and confocal microscope system (Nikon, Tokyo, Japan). pH measurements were made using a Mettler-Toledo Delta 320 pH meter (USA).

Synthesis of Spherical Nucleic Acids (SNAs)

Citrate-stabilized gold nanoparticles were prepared by the method developed by Michael J. Natan et al.[2] To form DNA-AuNP conjugates, 2 µM thiol-modified DNA was added to a 10 nM solution of 13 ± 1 nm AuNPs and incubated for 16 hour at room temperature. Sodium dodecylsulphate (SDS), phosphate buffer, and sodium chloride (NaCl) were added sequentially to give final concentrations of 0.01% (w%), 10 mM, 0.1 M respectively. After 8 h, an additional aliquot of NaCl was added to achieve a [NaCl] of 0.3 M for all systems. Sonication was performed for 10 seconds between additions. The final mixture was allowed to gently mix overnight at room temperature. Unbound DNA sequences were washed away by three successive rounds of centrifugation (16,000 rcf, 20 min) and resuspension using phosphate buffered saline mixed with Tween 20 (PBST) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, 0.1% Tween 20). The concentration of the AuNPs-DNA was determined by measuring absorption at 524 nm ($\varepsilon = 2.7 \times 10^8$ $L \cdot mol^{-1} \cdot cm^{-1}$).

Preparation of the TPE SNA Probe

Complementary RNA or DNA were hybridized to 10 nM SNAs in PBST. The solution was heated to 70° C for 30 minutes and then allowed to cool down to room temperature overnight. Unbound DNA or RNA sequences were washed away by three successive rounds of centrifugation (16,000 rcf, 20 min), supernatant removal, and resuspension in PBST. The mixture was then incubated with 4 µM EBMVC at room temperature overnight to allow EBMVC to associate with the DNA/RNA or DNA/DNA duplex. Unassociated EBMVC molecules were removed using iterative centrifugation-resuspension cycles.

Measurement of Binding Parameters of EBMVC to Duplexes

The intrinsic binding constants (k) were obtained by titration monitoring fluorescence change. An excitation wavelength of 450 nm was used. The fluorescence intensity at 575 nm was monitored and analyzed using the Scatchard equation (1)[3]

$$r / c_f = Kn - Kr \quad (1)$$

where k is the binding constant, n is the number of dye sites per DNA/RNA, r is the ratio of the concentration of the binding dye to the concentration of DNA/RNA (in buffer), and Cf is the concentration of free dye. The concentration of the binding compound was calculated using equation (2)[4]

$$C_b = C_t [(F-F_0)/(F_{max}-F_0] \quad (2)$$

where ct is the total compound concentration, F is the observed fluorescence intensity at given DNA/RNA concentration, F_0 is the fluorescence intensity in absence of DNA/RNA, and F_{max} is the fluorescence intensity with excess DNA/RNA.

Gel Electrophoresis Analysis of RNase H Activity on EBMVC-bound DNA/RNA Duplex

RNase H assay buffer consisting of 50 mM Tris-HCl (pH 8.5), 75 mM KCl, 3 mM $MgCl_2$, and 2 mM GSH was first prepared as a stock solution. Samples containing 100 nM DNA/RNA or DNA/RNA/EBMVC were incubated with RNase H (0.01 U) for 3 h. The products were then electrophoresed using 12% polyacrylamide gel at 110 V for 1 h in 1 × TBE buffer. Gel image was carried out with a ChemiDoc XRS+ Imaging System (Bio-RAD).

Spectrophotometric Measurements

Fluorescence measurements of the SNA probe (0.2 nM) were conducted in RNase H assay buffer. Following the additions of different concentration of RNase H, the solution was incubated at

37℃ for 2 h. After the reaction, the fluorescence intensities were recorded at excitation wavelength of 450 nm with emission wavelength in the range of 480-750 nm.

Nuclease Activity Kinetics and Gel Electrophoresis Analysis

Fluorescence measurements were conducted in enzyme assay buffer. Two samples, the DNA/RNA SNA (0.2 nM), dsDNA SNA (0.2 nM), were each mixed with enzymes (0.5 U/mL). The fluorescence (excitation = 450 nm, emission = 573 nm) was measured continuously for 3 hours at 37 ℃ with a Tecan Spark 10M plate reader. After the reaction, 50 nM dsDNA was added to the solution of the SNA probes and incubated for 10 min. The fluorescence spectra were recorded at excitation wavelength of 450 nm with emission wavelength in the range of 480-750 nm. For polyacrylamide gel electrophoresis, the SNA probes were similarly incubated with the enzymes, but were treated by KCN in the presence of oxygen prior to gel loading to remove the gold nanoparticle core. The products were then electrophoresed using 12% polyacrylamide gel at 110 V for 1 h in 1× TBE buffer. Gel images were recorded on a ChemiDoc XRS+ Imaging System (Bio-RAD).

Stability of the SNA Probes

Fluorescence measurements of the SNA probe (0.2 nM) were conducted in RNase H assay buffer following the addition of different chemical species, including Cys (5 mM), GSH (5 mM), ATP (10 mM), glucose (10 mM), caspase-3 (1.5 nM), Cyt C (0.28 U/mL), H_2O_2 (100 μM), ClO^- (100 μM), and RNase H (0.5 U/mL). The mixtures were allowed to stir for 2 h, and dsDNA was added to the solution to give a concentration of 50 nM. After 10 min, fluorescence spectra were recorded at an excitation wavelength of 450 nm with emission wavelength in the range of 480-750 nm.

Cell Culture

HeLa, MCF-7, and HepG2 cells were cultured using high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (10,000 U/mL, 10,000 μg·mL^{-1}, Invitrogen). Normal liver cells (L02) were grown in RPMI 1640 medium (Thermo Scientific HyClone) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), and 100 U/mL penicillin. Cultures were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Cell Imaging

For cells treated with SNA probe, cells were incubated with 0.1 nM DNA/RNA or dsDNA probe in the cell growth medium for 2 h at 37 ℃. Then, the SNAs were removed by washing with PBS. For the RNase H inhibitor (EDTA/gentamycin)-treated group, HepG2 cells were cultured with gentamycin (200 μM) in cell growth medium overnight. Next, the cells were treated with the SNA probe (0.1 nM) in culture media containing EDTA (2 mM, another inhibitor for RNase H) for 2 h. Confocal images were obtained using 800 nm excitation and emission in the range of 520-580 nm on an Olympus FV1000-MPE multiphoton laser scanning confocal microscope with fs laser using 5 mW laser power. Z-scanning images were taken every 2 μm by scanning the samples across a defined section along the z-axis.

Flow Cytometry

For flow cytometry analysis, the cells were washed with PBS (pH 7.4) three times and detached from culture plate with a trypsin-EDTA solution. Next the suspended cell solution was centrifuged at 2000 g for 5 min and washed with PBS three times. Finally, the cells were resuspended in PBS for flow cytometric analysis on a Beckman Coulter Gallios instrument using 488 nm excitation.

ELISA RNase H Detection in Cell-Free Extracts

Cell-free extracts were prepared as follows. Cells (5×10^6) were harvested with trypsin and centrifuged at 1500 g for 2 min. Then, cells were washed 3 times with 10 mL of cold PBS, centrifuged, and resuspended in 0.5 mL of ice-cold cell lysis buffer (Cell Signaling) on ice for 5 min. Cells were then pulse-sonicated on ice 5 times, 5 s each. The cellular debris was centrifuged at 15,000g for 20 min at 4 ℃ and supernatants were collected and stored at −20 °C. ELISA for RNase H was carried out using a Human RNase H ELISA kit following manufacturer-suggested protocols on the cell-free extracts.

Tissue Imaging

Rat liver tissue slices were treated with PBS or EDTA/gentamycin for 3 h before incubation with the SNA probes (with DNA/RNA or dsDNA) for another 3 h. Thereafter, the tissues were washed with PBS 3 times to remove free SNAs, followed by two-photon imaging of the tissues using a Nikon A1R MP multiphoton and confocal microscope system (Nikon, Tokyo, Japan).

RNase H Analysis in HIRI Rat Groups

All Sprague-Dawley rats were fasted for 12 h before the experiment but were given access to water. Nine Sprague-Dawley rats were randomly divided into 3 equal groups: the sham-operated group (Sham), the IR group (IR), and the IR + octreotide pretreatment group (OCTIR). Each rat was anesthetized with an intraperitoneal injection of 10% chloral hydrate (30 $mg{\cdot}kg^{-1}$). Thirty minutes before laparotomy, the OCTIR group rats received an injection of octreotide intraperitoneally (20 $\mu g{\cdot}kg^{-1}$, Jilin Province A-Think Pharmaceutical Co., Ltd., Jilin, China). Control (Sham) and IR group rats received the same volume isotonic saline. All rats underwent a laparotomy. HIRI models were set up in the IR and OCTIR groups by using the Pringle's method,[5] which consists of ischemia for 0.5 h followed by reperfusion for 4 h. All rats were sacrificed, and the liver tissue slices (1-2 mm) were collected.

For fluorescence imaging assay, liver tissue slices (1-2 mm) were immersed in a PBS solution containing the SNA probe (0.2 nM) at 37 ℃ for 3 h. Thereafter, washing with PBS was carried out to remove free SNAs, followed by two photon imaging of the tissues using a Nikon A1R MP multiphoton and confocal microscope system (Nikon, Tokyo, Japan).

ELISA RNase H Detection in Tissue Homogenates.

The tissues were first rinsed with ice-cold PBS (0.01M, pH 7.4) to remove excess blood thoroughly. Tissues were weighed and then minced to small pieces, which were homogenized in PBS (the volume depends on the weight of the tissue; 9 mL PBS was added to every 1 gram of tissue) with a glass homogenizer on ice. To further break the cells, the suspension was sonicated with an ultrasonic cell disrupter. The homogenates were then centrifuged for 5 minutes at 5000g, and supernatants were collected and analyzed using a rat RNase H ELISA kit following manufacturer-suggested protocol.

Additional Tables and Figures

Table S1. Oligonucleotides used in this work.

Name	Base sequence
G-rich	5’-GGGTAGGGCGGGTTGGGT-3’
Poly A	5’-AAAAAAAAAAAA-3’
Poly C	5’-CCCCCCCCCCCC-3’
Poly T	5’-TTTTTTTTTTTT-3’
dsDNA	3’-CATGAGTTCCTAAGACTACTG-5’ 5’-GTACTCAAGGATTCTGATGAC-3’
DNA/RNA	3’-AUGAGUUCCUAAGACUACUG-5’ 5’-GTACTCAAGGATTCTGATGAC-3’
ssDNA	5’-GTACTCAAGGATTCTGATGAC-3’
RNA	5’-GUCAUCAGAAUCCUUGAGUA-3’
DNA-thiol	5’-GTACTCAAGGATTCTGATGAC-thiol-3’
TAMRA-DNA-thiol	5’-TAMRA-GTACTCAAGGATTCTGATGAC-thiol-3’

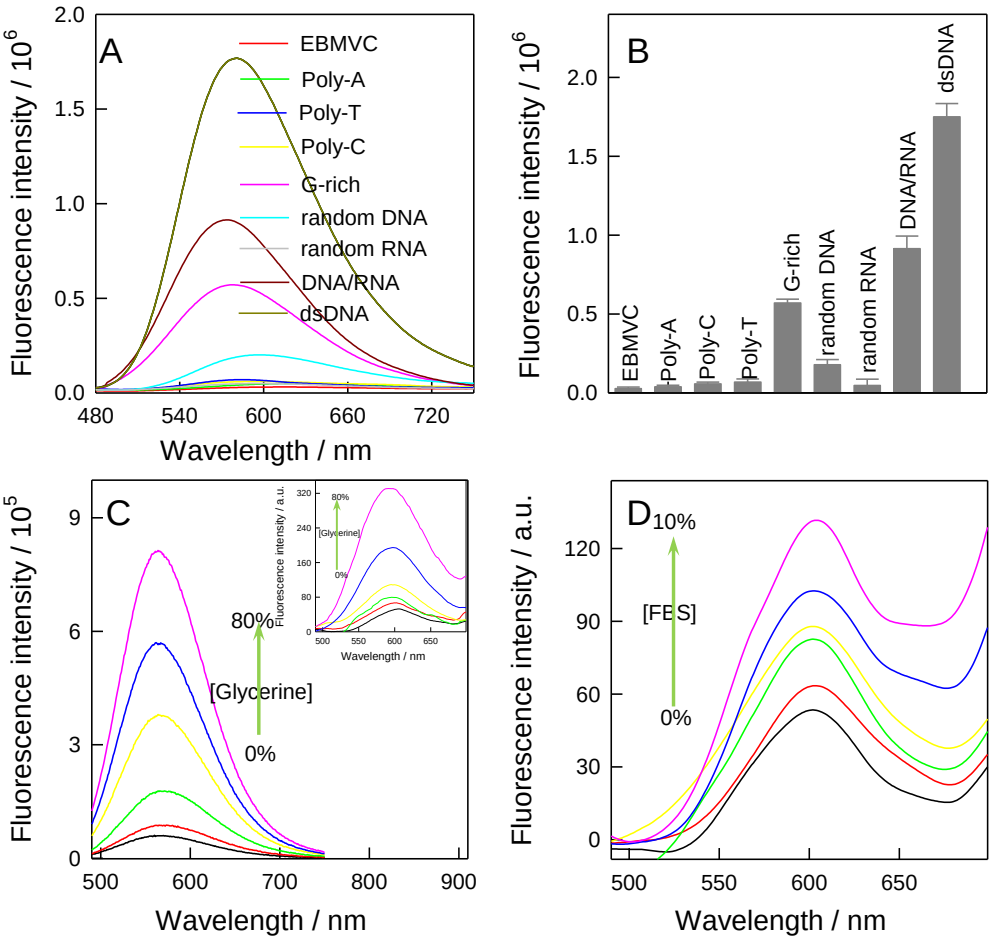

Fig. S1 (A),(B) The fluorescence enhancement of EBMVC (500 nM) with different forms of nucleic acids (100 nM). $\lambda_{ex}/\lambda_{em}$ = 450 nm/573 nm. (C) Fluorescence enhancement of EBMVC with different concentrations of Glycerine, λ_{ex}= 450 nm. Inset: Fluorescence spectra excited with two photon, λ_{ex}= 800 nm. (D) Fluorescence enhancement of EBMVC with different concentrations of FBS, excited with two photon, λ_{ex}= 800 nm.

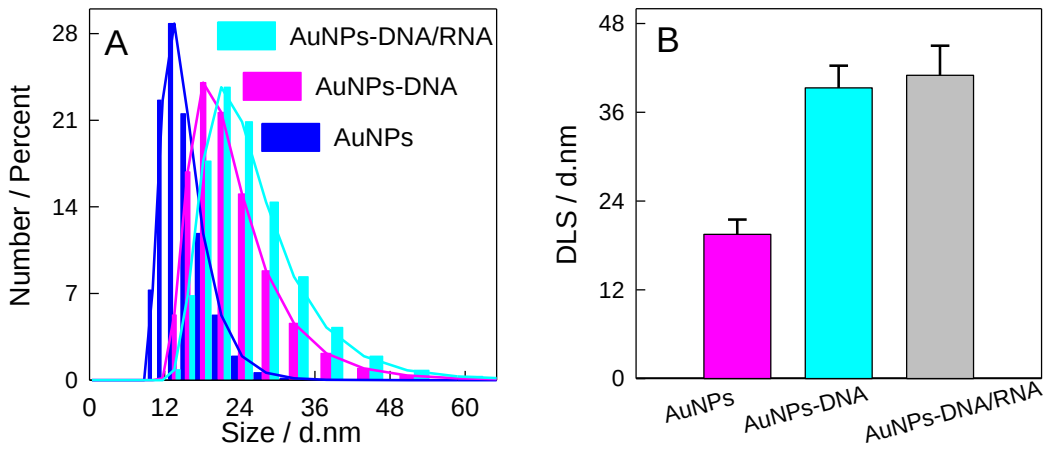

Fig. S2 Number-average size distribution (A) and Z-average size (B) measured by dynamic light scattering.

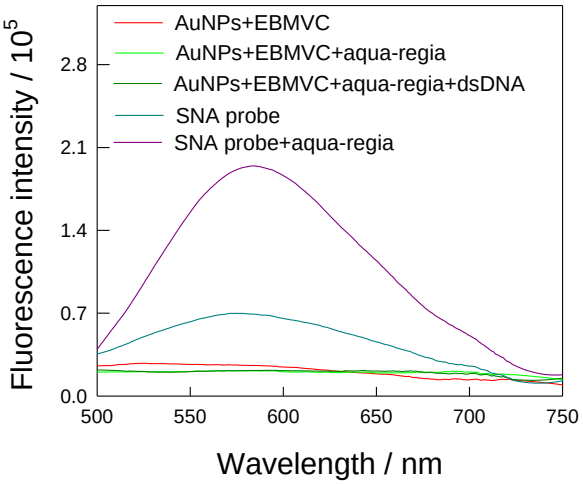

Fig. S3 Fluorescence spectra of the SNA probe and controls upon addition of aqua regia.

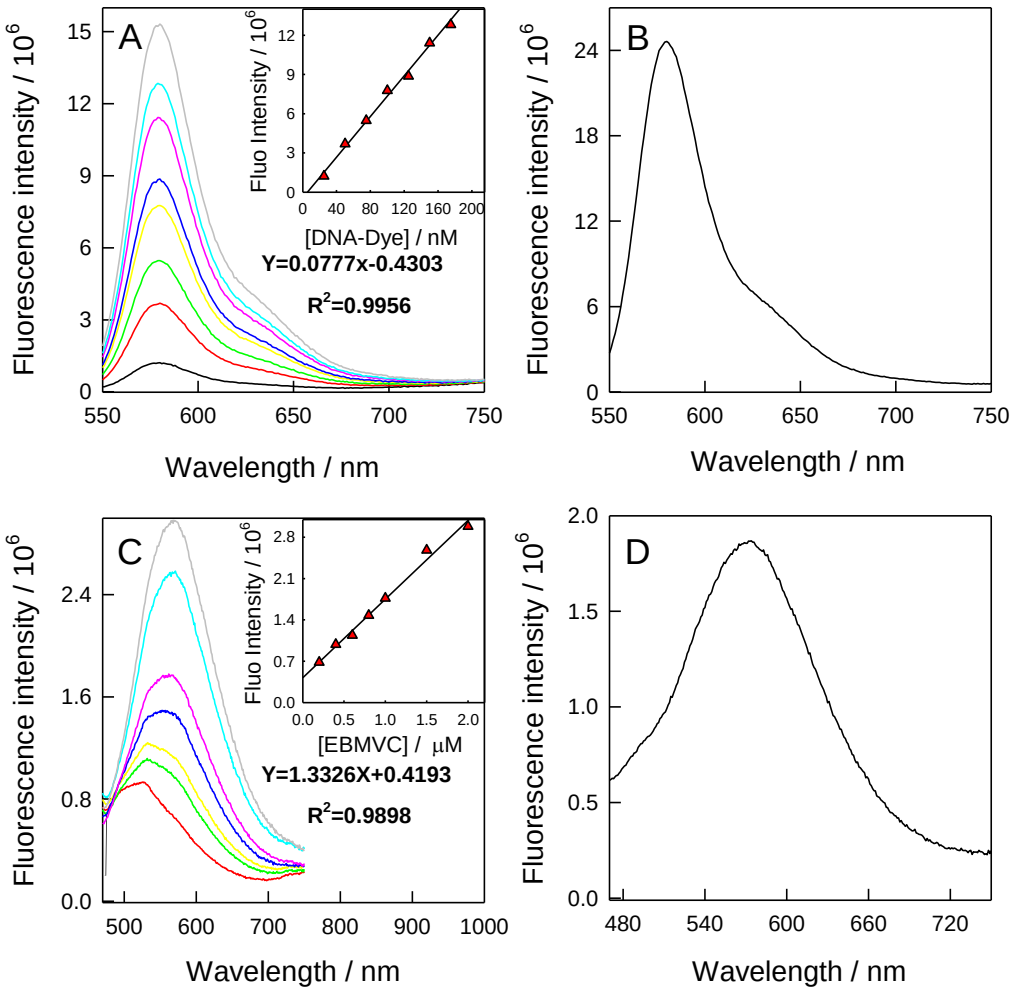

Fig. S4 Fluorescence-concentration standard curve for TAMRA-DNA (A) and fluorescence spectrum of TAMRA-DNA obtained from the SNA probe with treatment of KCN and O_2 (B). (C) Fluorescence-concentration standard curve for EBMVC. (D) Fluorescence spectrum of EBMVC remaining in the supernatant after loading onto DNA/RNA SNA probe. The value is used calculate the loading efficiency.

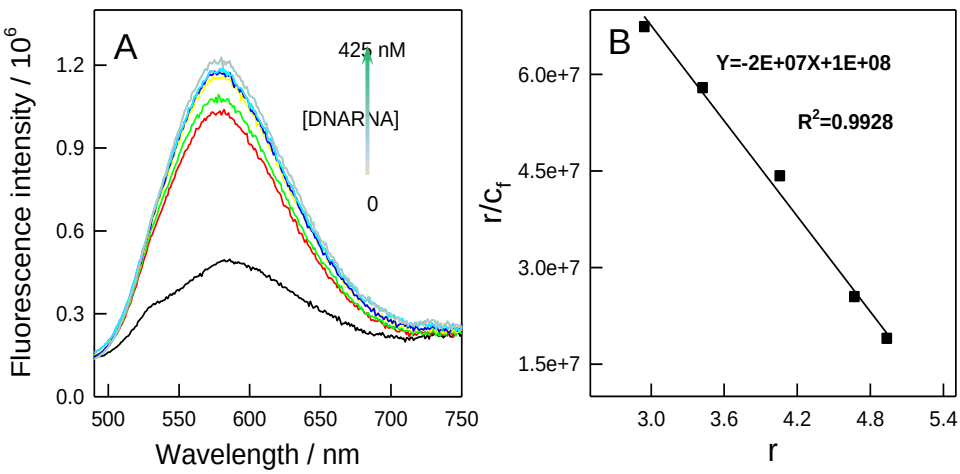

Fig. S5 (A) Titration of EBMVC with DNA/RNA and (B) curve fitting according to Scatchard equation. λex = 450 nm and λem= 573 nm. [EBMVC] = 1000 nM, [DNA/RNA] = 0 ~ 425 nM. Calculated binding constant: 2×10^7 M^{-1}. Goodness-of-fit: R^2 =0.9928

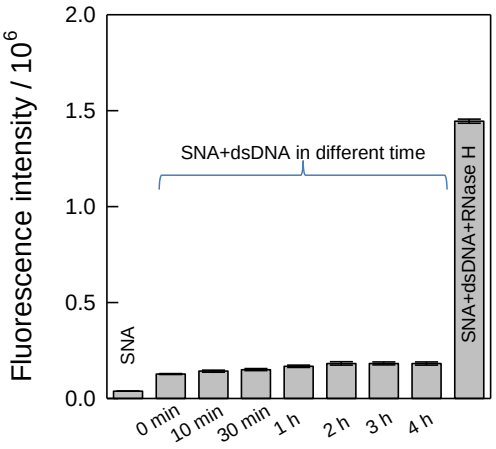

Fig. S6 Stability of the SNA probe in the presence of dsDNA. Bars represent the fluorescence intensity of DNA/RNA SNA in buffer solution treated with or without dsDNA in diffenent time.

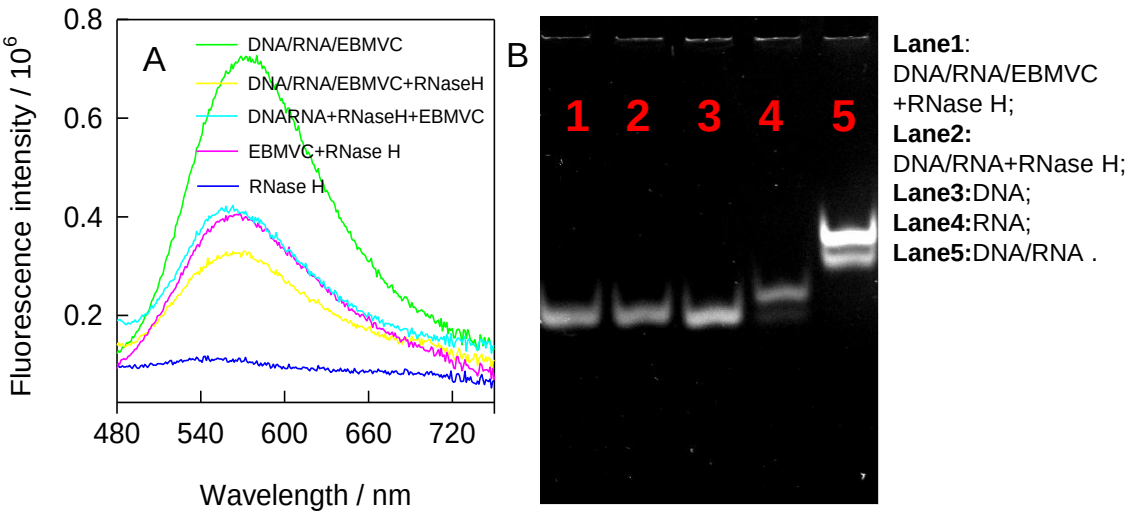

Fig. S7 A) Fluorescence emission spectra of DNA/RNA/EBMVC with/without RNase H treatment. B) Polyacrylamide gel electrophoresis, showing the disappearance of the RNA band upon treatment with RNase H for both the DNA/RNA duplex and the duplex loaded with EBMVC.

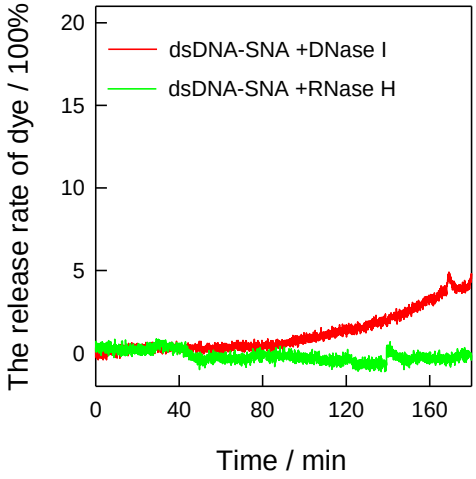

Fig. S8 Nuclease degradation kinetics of the dsDNA SNA probe. The release rate of dye from the dsDNA SNA probe (0.2 nM) with addition of 0.5 U/mL RNase H or DNase I were monitored over time.

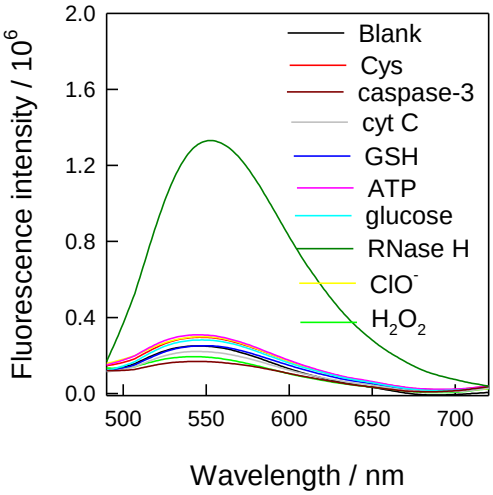

Fig. S9 Stability of the SNA probe at 37 ℃ to a panel of cellular species: Cys (5 mM), GSH (5 mM), ATP (10 mM),glucose (10 mM), caspase-3 (1.5 nM), Cyt C (0.28 U/mL), H2O2 (100 μM), ClO- (100 μM), and RNase H (0. 5 U/mL).

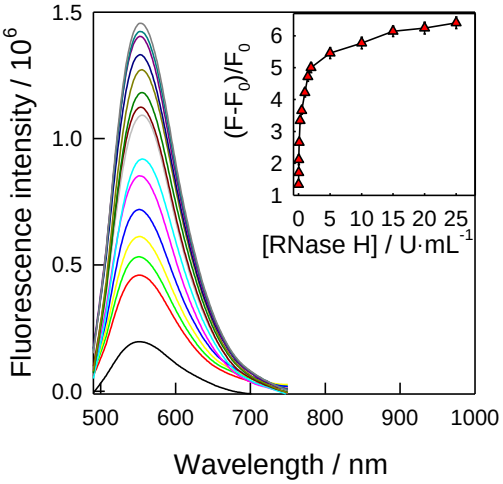

Fig. S10 Titration of SNA probes using RNase H in the presence of dsDNA. Error bars are obtained from three parallel samples.

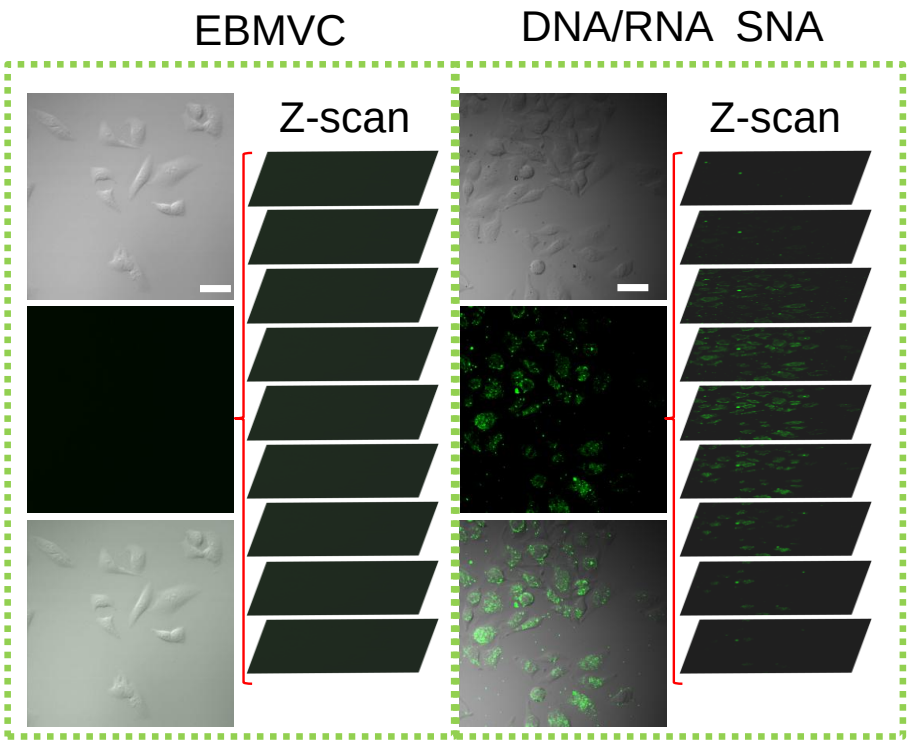

Fig. S11 Two-photon excitation confocal fluorescence microscopy images of HepG2 cells after incubation with EBMVC (200 nM) or SNA probe (0.1 nM). Excitation: 800 nm using fs laser pulses (5 mW laser power). Scale bar: 50 μm.

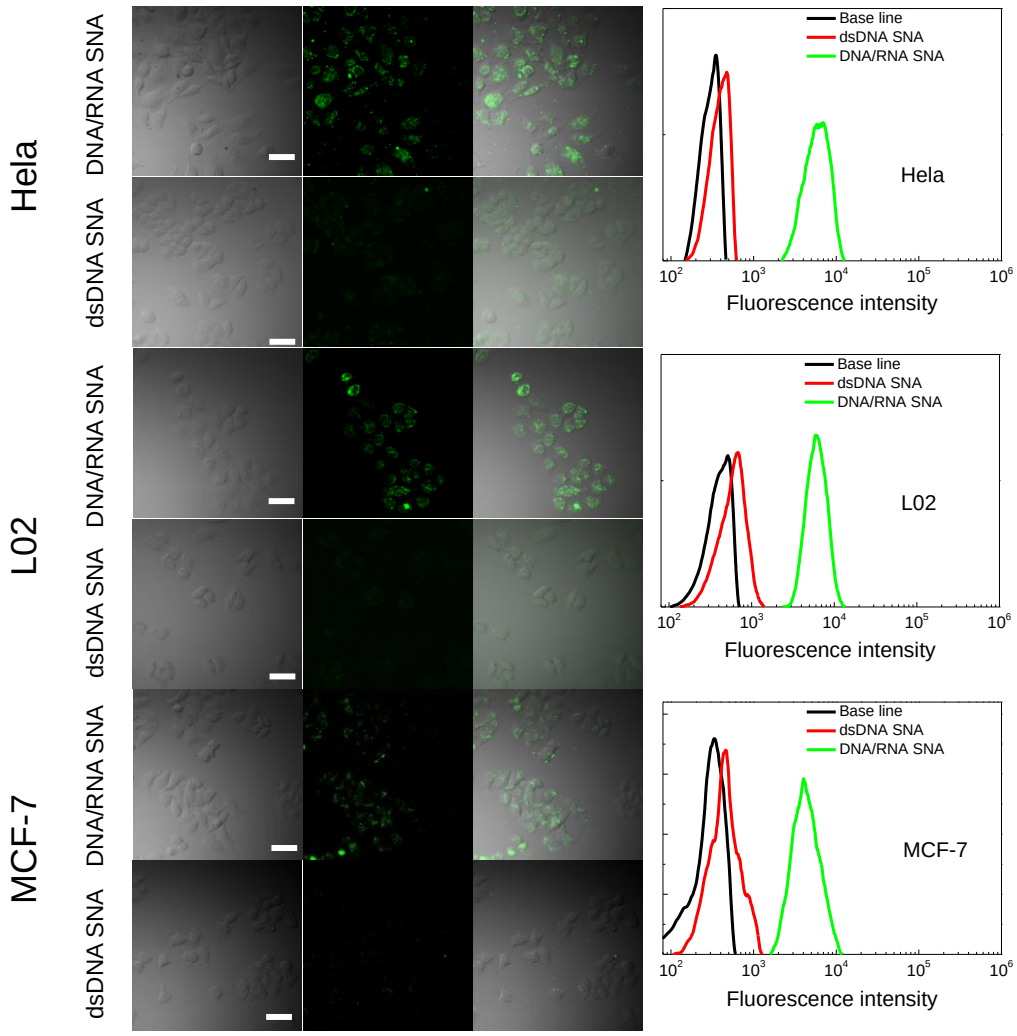

Fig. S12 Confocal images and flow cytometry analyses of different cell lines treated with the DNA/RNA SNA probe or dsDNA SNA probe. Excitation: 800 nm. Scale bars: 50 μm.

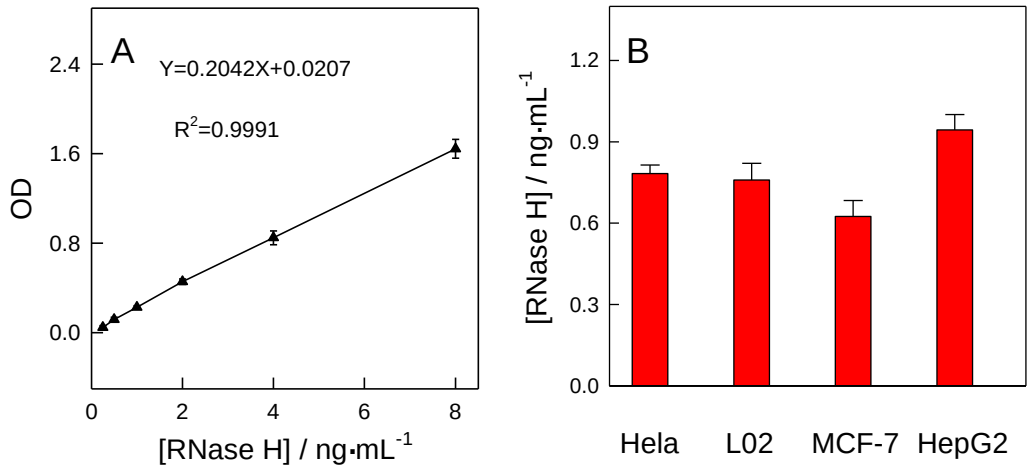

Fig. S13 Quantification of RNase H levels in different cell lines using ELISA. A) standard curve for RNase H; B) RNase H levels in different cell-free extracts. Error bars were calculated from three independent experiments.

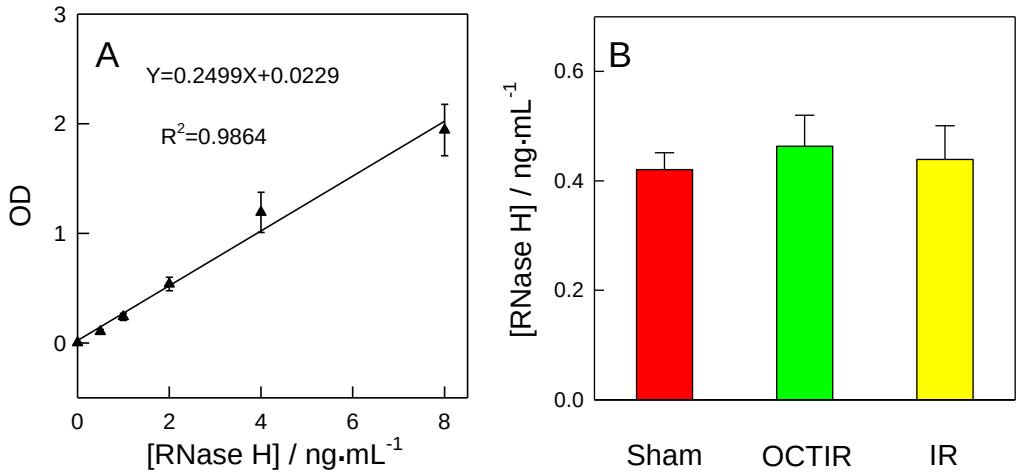

Fig. S14 Quantification of RNase H levels in rat liver tissues using ELISA. A) standard curve for RNase H, B) RNase H levels in three group rat liver tissues homogenates. Error bars were calculated from samples from three different rats.

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