Electronic Supplementary Information (ESI)

A Supersandwich Fluorescence *in Situ* Hybridization (SFISH) Strategy for Highly Sensitive and Selective mRNA Imaging in Tumor Cells

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EXPERIMENTAL SECTION

1. DNA and RNA Sequences

Table S1. The DNA sequences used in the work (Sangon Biological Co. Ltd**)**:

Names	Sequences(5'-3')
For TK1 mRNA:	
Hairpin Probe 1 (HP1)	TCTGTTCTATCGAACCAGGGAGAACAGAAACTCA
Signal Probe 1 (SP1)	FAM-TACTCCAGGTGCACGATAGAACAGA
Signal Probe 2 (SP2)	GCACCTGGAGTAATCTGTTCTATCG-FAM
For Survivin mRNA:	
Hairpin Probe 2 (HP2)	CACCGCATCAAAGTTAGAGATGCGGTGGTC
Signal Probe 3 (SP3)	Cy5-TGTCGTCAAGATATTTGATGCGGTG
Signal Probe 4 (SP4)	ATCTTGACGACAACACCGCATCAAA-Cy5
For ND6 mRNA:	
Hairpin Probe 3 (HP3)	TACTGGTGTTTTGCCAATGTTCACCAGTAACTTCA
Signal Probe 5 (SP5)	FAM-AACTACACCTGCGAAAACACCAGTA
Signal Probe 6 (SP6)	GCAGGTGTAGTTGTACTGGTGTTTT-FAM
For qRT-PCR:	
TK1 forward:	CTCCTACCCACTGGTCTGCTTA
TK1 reverse:	CAGGGAGAACAGAAACTCAGCA
GAPDH forward:	TGGGTGTGAACCATGAGAAGT
GAPDH reverse:	TGAGTCCTTCCACGATACCAA
Inhibitor for TK1	CCAGGGAGAACAGAAAC
Control hairpin	CGAACAGGTGGGTGGGGGGGTGGATTGTTCG

Table S2. The RNA targets used in the work: Adopted from NCBI database.

The part of TK1 mRNA (red part denotes the selected target sequence):
GUGUGGCUGCCCCACCUGCCGCAUGCUCCCUCCUCUCCUACCCACUGGU
CUGCUUAAAGCUUCCCUCUCAGCUGCUGGGACGAUCGCCCAGGCUGGAG
CUGGCCCCGCUUGGUGGCCUGGGAUCUGGCACACUCCCHUCUCCUUGGG
GUGAGGGACAGAGCCCCACGCUGUUGACAUCAGCCUGCUUCUUCCCCUC
UGCGGCUUUCACUGCUGA**GUUUCUGUUCUCCCUGG**GAAGCCUGUGCCA
GCACCUUUGAGCCUUGGCCCACACUGAGGCUUAGGCCUCUCUGCCUGGG
AUGGGCUCCCACCCUCCCCUGAGGAUGGCCUGGAUUCACGCCCUCUUGU
UUCCUUUUGGGCUCAAAGCCCUUCCUACCUCUGGUGAUGGUUUCCACAG
GAACAACAGCAUCUUUCACCAAGAUGGGUGGCACCAACCUUGCUGGGA
CUUGGAUCCCAGGGGCUUAUCUCUUCAAGUGUGGAGAGGGCAGGGUCC
ACGCCUCUGCUGUAGCUUAUGAAAUUAACUAAUUGAAAAUUCACUGGU
The part of Survivin mRNA (red part denotes the selected target sequence):
AUGGGUGCCCCGACGUUGCCCCCUGCCUGGCAGCCCUUUCUCAAG**GACC**
ACCGCAUCUCUACAUUCAAGAACUGGCCCUUCUUGGAGGGCUGCGCCU
GCACCCCGGAGCGGAUGGCCGAGGCUGGCUUCAUCCACUGCCCCACUGA
GAACGAGCCAGACUUGGCCCAGUGUUUCUUCUGCUUCAAGGAGCUGGA
AGGCUGGGAGCCAGAUGACGACCCCAUGAGAGAGCUCUGUUAGCAGAA
UGAAAAAAUUGGAAGCCAGAUUCAGGGAGGGACUGGAAGCAAAAGAAU
UUCUGUUCGAGGAAGAGCCUGAUGUUUGCCAGGGUCUGUUUAACUGGA
CAUGAAGAGGAAGGCUCUGGACUUUCCUCCAGGAGUUUCAGGAGAAAG
The part of MT-ND6 mRNA (red part denotes the selected target sequence):
AUGAUGUAUGCUUUGUUUCUGUUGAGUGUGGGUUUAGUAAUGGGGUUU
GUGGGGUUUUCUUCUAAGCCUUCUCCUAUUUAUGGGGGUUUAGUAUUG
AUUGUUAGCGGUGUGGUCGGGUGUGUUAUUAUUCUGAAUUUUGGGGGA
GGUUAUAUGGGUUUAAUAGUUUUUUUAAUUUAUUUAGGGGGAAUGAU
GGUUGUCUUUGGAUAUACUACAGCGAUGGCUAUUGAGGAGUAUCCUGA
GGCAUGGGGGUCAGGGGUUGAGGUCUUGGUGAGUGUUUUAGUGGGGUU
AGCGAUGGAGGUAGGAUUGGUGCUGUGGGUGAAAGAGUAUGAUGGGGU
GGUGGUUGUGGUAAACUUUAAUAGUGUAGGAAGCUGAAUAAUUUAUGA
AGGAGAGGGGUCAGGGUUGAUUCGGGAGGAUCCUAUUGGUGCGGGGGC
UUUGUAUGAUUAUGGGCGUUGAUUAGUAGU**AGUUACUGGUGAACAUU**
GUUUGUUGGUGUAUAUAUUGUAAUUGAGAUUGCUCGGGGGAAUAGG

2. Cell Culture and Fixation

HepG2 cells (human liver hepatocellular carcinoma cell line), MCF-7 cell（a breast cancer cell line）and L02 cells (human hepatocyte cell line) were cultured in an RPMI 1640 medium supplemented with 15% fetal calf serum, 100 μg mL^{-1} of streptomycin, and 100 units mL^{-1} of penicillin. Cells were all cultured at 37 °C in a humidified incubator containing 5% CO_2. HepG2, MCF-7 and L02 cells were seeded on confocal laser culture slides and cultured in the culture medium for 24 h. The cells were first washed twice with phosphate buffered saline (PBS, pH 7.4, calcium and

magnesium free) and then fixed on the slides with PBS containing 4 % paraformaldehyde (PFA) for 15 min at room temperature followed by two PBS washes.

3. Procedure of SFISH and FISH

Taking TK1 mRNA (Table S1) for example, the slides with fixed cells were first incubated in a humidified 37 °C incubator for 1.5 h with 50 μL of hybridization solution containing 30 μL of 5 μM HP1, 10 μL of formamide, and 10 μL of 10 × SSC. After being washed twice with 2 × SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. If using procedure of SFISH, a hybridization solution containing 140 μL of 3 μM FAM-labeled SP1 and SP2, 2 × SSC, and 20% formamide was added to the slides and incubated in a humidified 37 °C incubator for 2 h. If using procedure of FISH, a hybridization solution containing only 140 μL of 3 μM FAM-labeled SP1, 2 × SSC, and 20% formamide was used. Then the slides were washed with PBS twice for 3 min each. Before imaging, the slides were stained with 30 nM DAPI solution for 10 min and then washed twice with PBS. In addition, HP2, SP3 and SP4 were used for Survivin mRNA (Table S1) detection, and HP3, SP5 and SP6 were used for ND6 mRNA (Table S1) assay.

4. Inhibitor and Drug Treated for TK1 mRNA

The slides with fixed cells were first incubated in a humidified 37 °C incubator for 1.5 h with 50 μL hybridization solution containing 30 μL of 5 μM Inhibitor, 10 μL of formamide, and 10 μL of 10 × SSC. After being washed twice with 2 × SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. Then the cells were incubated for 1.5 h with 50 μL of hybridization solution containing 30 μL of 5 μM HP1, 10μL of formamide, and 10 μL of 10 × SSC. After being washed twice with 2 × SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. A hybridization solution containing FAM-labeled SP1，SP2, 2 × SSC, and 20% formamide (140 μL) was added to the slides and incubated in a humidified 37 °C incubator for 2h. The slides were washed with PBS twice for 3 min each. Before imaging, the slides were stained with 30 nM DAPI solution for 10 min and then washed twice with PBS. For the drug treatment, HepG2 cells were seeded on glass slides overnight. Then the slides were first incubated with 5 μM tamoxifen or 10 nM β-Estradiol in a humidified 37 °C and 5% CO_2 incubator for 24 h and followed the above experimental steps.

5. Dual-Color in situ Detection of TK1 and Survivin mRNA

The slides with fixed cells were first incubated in a humidified 37 °C incubator for 1.5 h with 100 μL of hybridization solution containing 30 μL of 5 μM HP1 and 30 μL of 5 μM HP2, 20 μL of formamide, and 20 μL of 10 × SSC. After being washed twice with 2 × SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. A hybridization solution containing 280 μL of FAM-labeled SP1 and SP2, Cy5-labeled SP3 and SP4, 2 × SSC, and 20%

formamide was added to the slides and incubated in a humidified 37 °C incubator for 2h. The slides were washed with PBS twice for 3 min each. Before imaging, the slides were stained with 30 nM DAPI solution for 10 min and then washed twice with PBS.

6. Fluorescene Imaging of ND6 mRNA mutation

The slides with cells were added prewarmed (37 °C) staining solution containing MitoTracker probe first, then incubated in a humidified 37 °C incubator for 40 min. Carefully remove the staining solution covering the cells, and replace it with PBS containing 4% paraformaldehyde (PFA) for 15 min at room temperature followed by two PBS washes. The slides with fixed cells were first incubated in a humidified 37 °C incubator for 1.5 h with 50 μL of hybridization solution containing 30 μL of 5 μM HP3, 10 μL of formamide, and 10 μL of 10× SSC. After being washed twice with 2× SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. A hybridization solution containing 140 μL of 3 μM FAM-labeled SP5 and SP6, 2× SSC, and 20% formamide was added to the slides and incubated in a humidified 37 °C incubator for 2 h. Then the slides were washed with PBS twice for 3 min each. Before imaging, the slides were stained with 30 nM DAPI solution for 10 min and then washed twice with PBS.

7. Detection of TK1 mRNA in Tissue Slices

The tissue slices were first incubated in a humidified 37 °C incubator for 1.5 h with 300 μL of hybridization solution containing 180 μL of 5 μM HP1, 60 μL of formamide, and 60 μL of 10× SSC. After being washed twice with 2× SSC, the tissue slices were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. A hybridization solution containing 840 μL of 3 μM FAM-labeled SP1 and SP2, 2× SSC, and 20% formamide was added to the slides and incubated in a humidified 37 °C incubator for 2 h. Then the tissue slices were washed with PBS twice for 3 min each. Before imaging, the tissue slices were stained with 30 nM DAPI solution for 10 min and then washed twice with PBS.

8. Confocal Fluorescence Imaging System

Fluorescence imaging was performed using a confocal laser scanning fluorescence microscope setup consisting of an Olympus IX-70 inverted microscope with an Olympus FluoView 500 confocal scanning system. The cellular images were acquired using a 120× objective. An Ar^+ laser (488 nm) was used as an excitation source for a FAM-labeled detection probe, and a 515 nm (±10 nm) bandpass filter was used for fluorescence detection. A red He Ne laser (633 nm) was used as an excitation source for the Cy5-labeled probe, and a 660 nm longpass filter was used for fluorescence detection. The DAPI dye was excited with a 405 nm laser line and detected with a 460 nm (±10 nm) bandpass filter. The fluorescence images were presented after processing by image proplus 6.0 software and Image J version 1.38x software.

9. qRT-PCR and Flow Cytometry Assay for Cellular TK1 mRNA

For qRT-PCR, total cellular RNA was extracted from HepG2 cells, L02 cells or

MCF-7 cells using Trizol reagent S5 (Sangon Co.Ltd., Shanghai, China) according to the indicated protocol. The cDNA samples were prepared by using the reverse transcription (RT) reaction with AMV First Strand cDNA Synthesis Kit (BBI, Toronto, Canada). qRT-PCR analysis of mRNA was performed with SG Fast qPCR Master Mix(2X) (BBI), according to the indicated protocol on an LightCycler480 Software Setup (Roche). The primers used in this experiment were shown in Table S1. We evaluated all of the data with respect to the mRNA expression by normalizing to the expression of GAPDH and using the $2^{-\Delta\Delta Ct}$ method.

For flow cytometry, cells were treated as above described, and then were analyzed by flow cytometry on a Beckman Gallios (Beckman Coulter, Brea, CA, USA) using fluorescent channel FL-1 (488 nm excitation, 525 nm emission). Reported fluorescent intensities represent the median of 20,000 analyzed cells. Background fluorescence detected in cells without staining was subtracted.

Supporting Figures

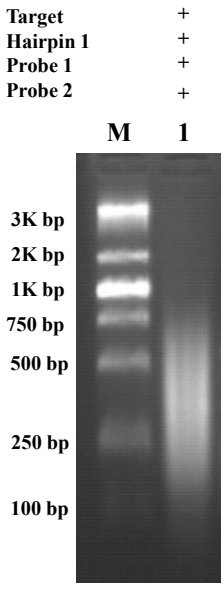

Figure S1. Electrophoresis of supersandwich product: Agarose gel (2%) electrophoresis of DNA ladder (left lane) and the supersandwich product (right lane).

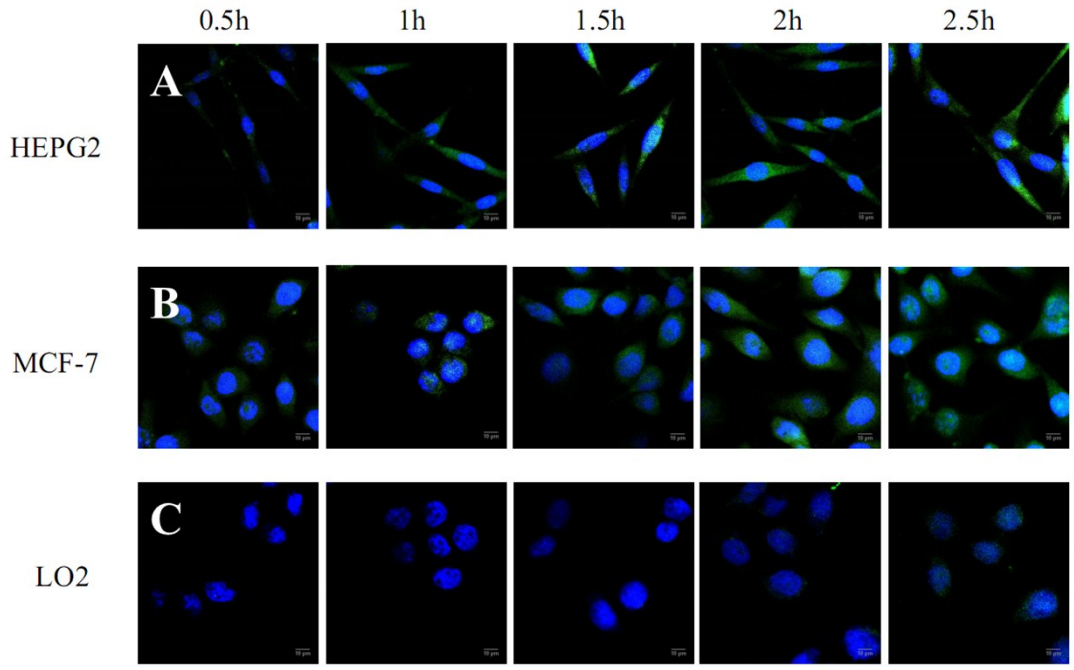

Figure S2. Fluorescence images of time optimization: SFISH detection of TK1 mRNA in HEPG2 (A), MCF-7 (B) and LO2 (C) cells in different time. The green fluorescence represents a FAM-labeled signal probes (PMT 780 V, gain 2%, and offset 10%), and the blue fluorescence is DAPI stained cell nuclei.

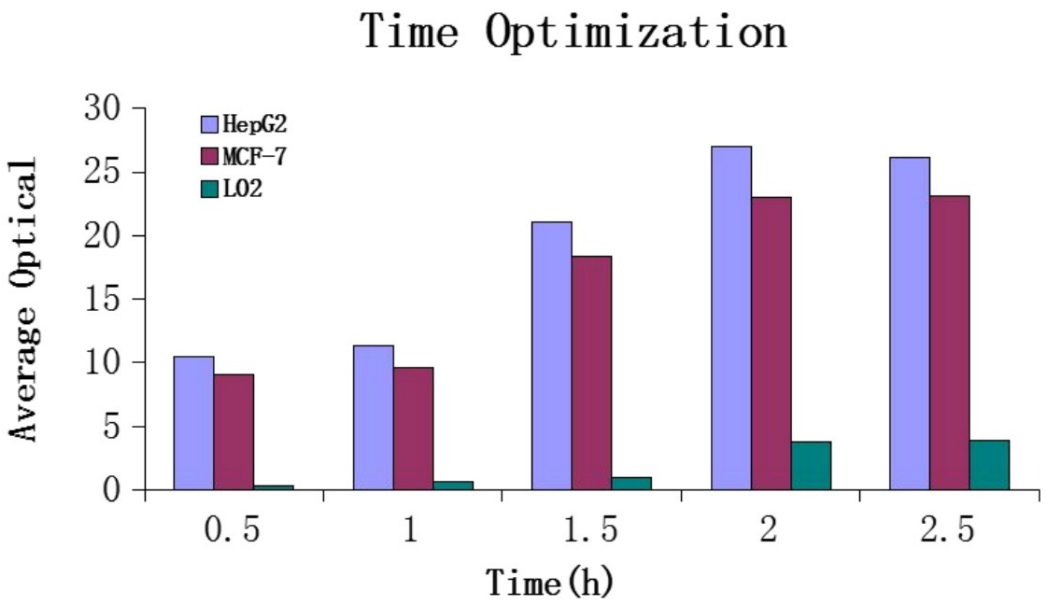

Figure S3. The histogram of time optimization: The mean fluorescence intensity of the three cell lines against different time. (The data come from Figure S2)

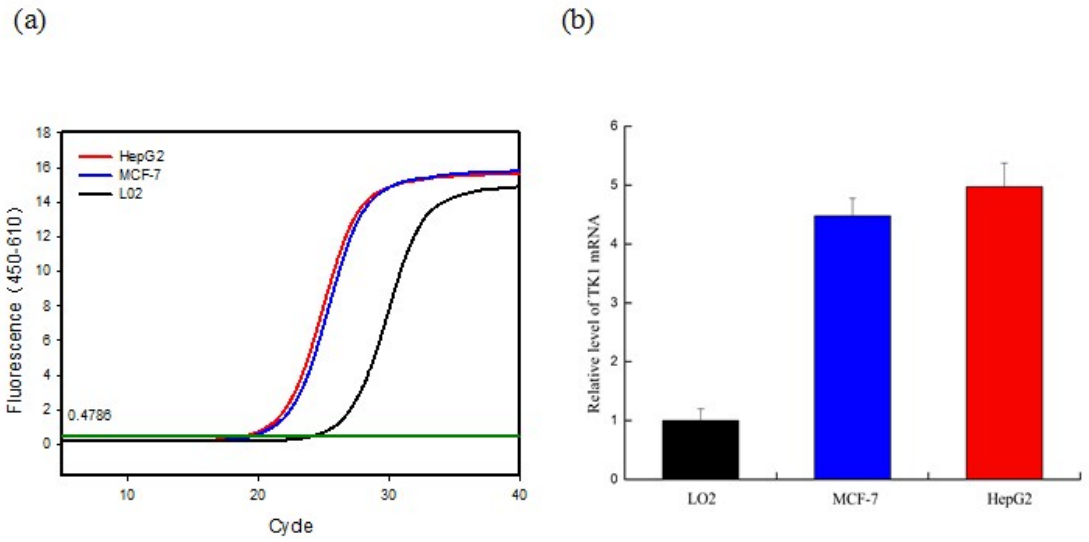

Figure S4. TK1 mRNA analysis by qRT-PCR: Analysis of TK1 mRNA in three kinds of cell lines, HepG2, MCF-7 and L02, by qRT-PCR. (a) Real-time fluorescence curves in qRT-PCR analysis. The green horizontal line represents the threshold line and the number on the lines indicates the threshold value. (b) Relative expression levels for TK1 mRNA in the three different cell lines.

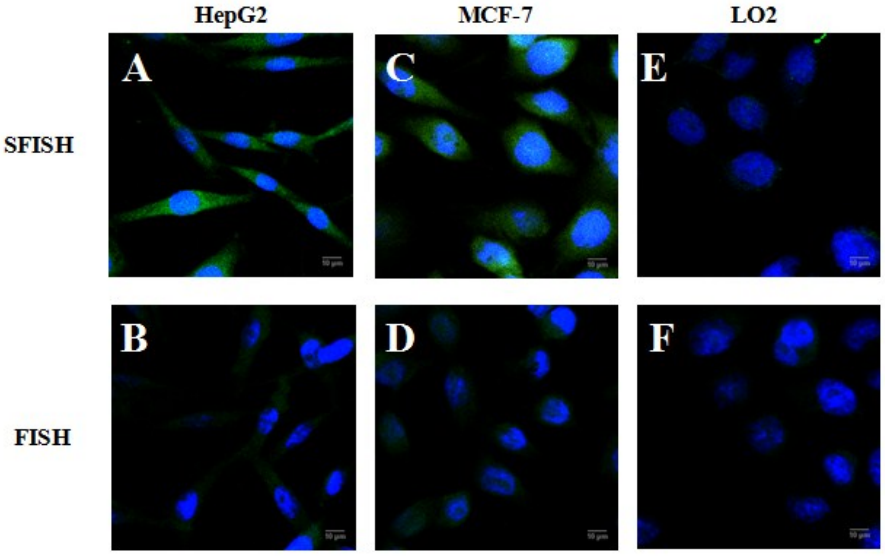

Figure S5. Signal amplification anlysis by fluorescence images: Visualization of TK1 mRNA in HepG2 cells, MCF-7 cells and L02 cells through SFISH (A, C, E) and traditional FISH (B, D, F), respectively. The green fluorescence represents a FAM-labeled signal probes and the blue fluorescence is DAPI stained cell nuclei.

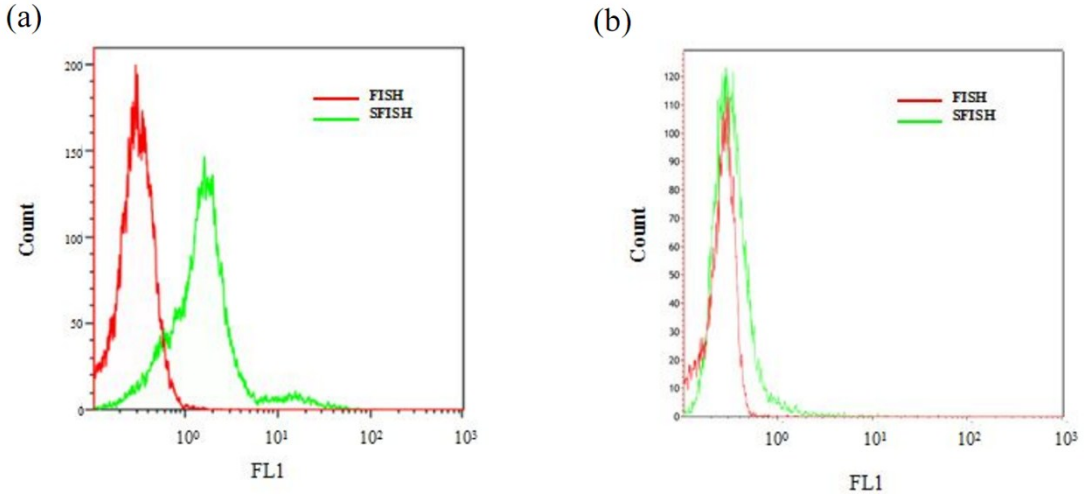

Figure S6. Signal amplification anlysis by flow cytometry: Flow cytometry analysis of HepG2 cell line (a) and L02 cell line (b) by detection of TK1 mRNA through FISH and SFISH techniques, respectively.

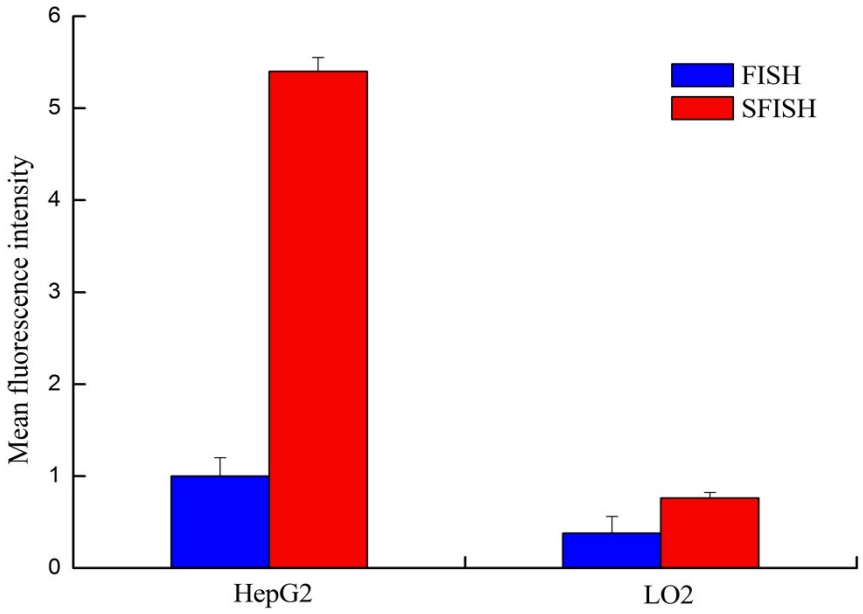

Figure S7. The normalized histogram of signal amplification anlysis by flow cytometry: The normalized histogram of flow cytometry analysis of HepG2 cell line and L02 cell line by detection of TK1 mRNA through FISH and SFISH techniques, respectively. The original data could be seen in Figure S6.

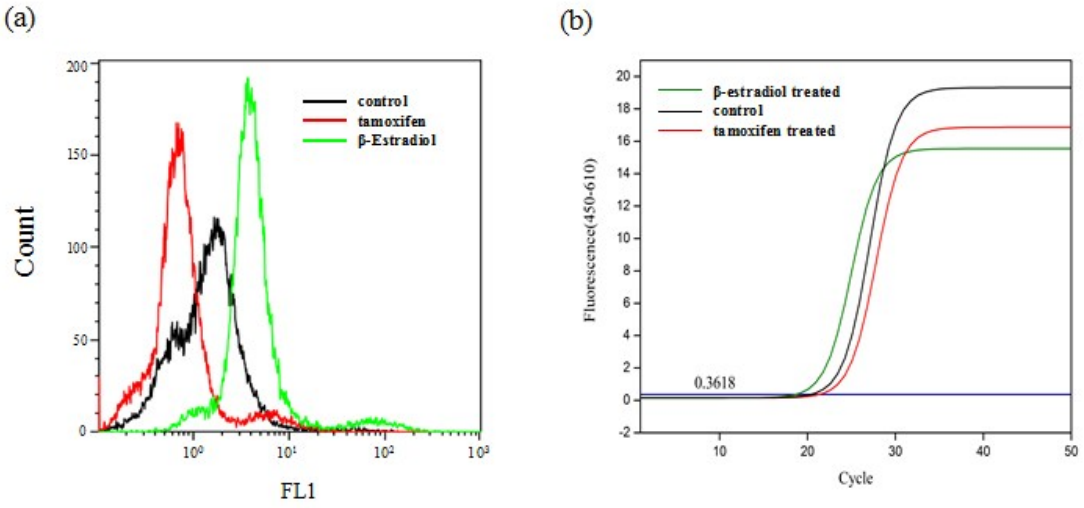

Figure S8. Flow cytometry and qRT-PCR analysis for drug stimuli: Analysis of TK1 mRNA in HepG2 cells in the three test groups (tamoxifen treated, β-estradiol treated and control) by (a) flow cytometry, and (b) qRT-PCR.

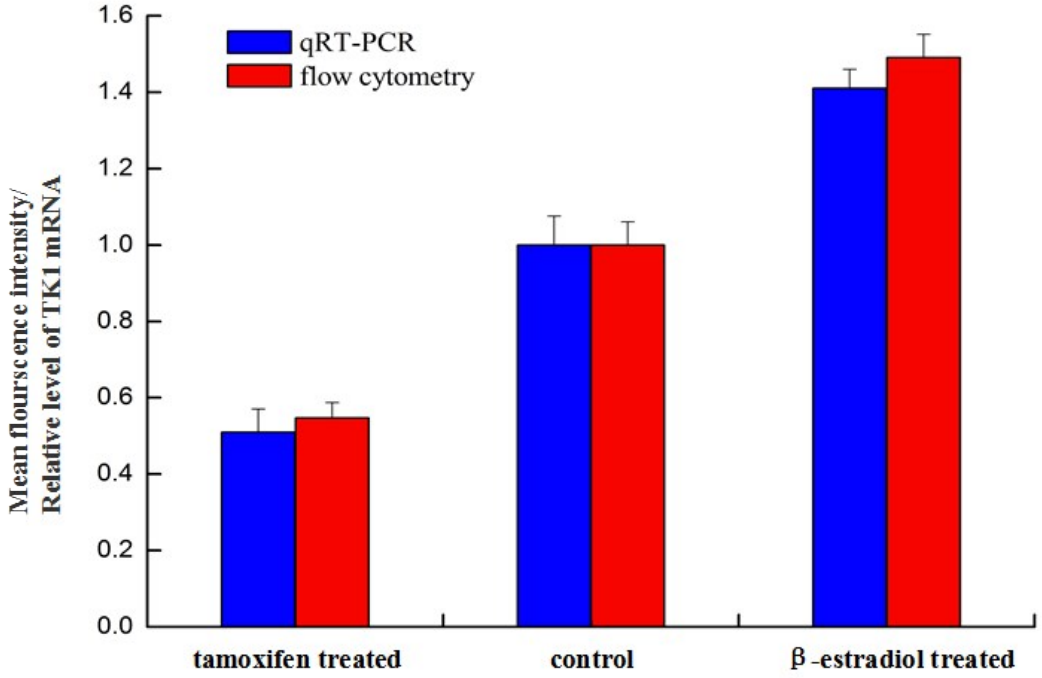

Figure S9. The normalized histogram of flow cytometry and qRT-PCR analysis for drug stimuli: The normalized histogram of qRT-PCR and flow cytometry analysis of TK1 mRNA in HepG2 cells in the three test groups (tamoxifen treated, β-estradiol treated and control). The original data could be seen in Figure S8.

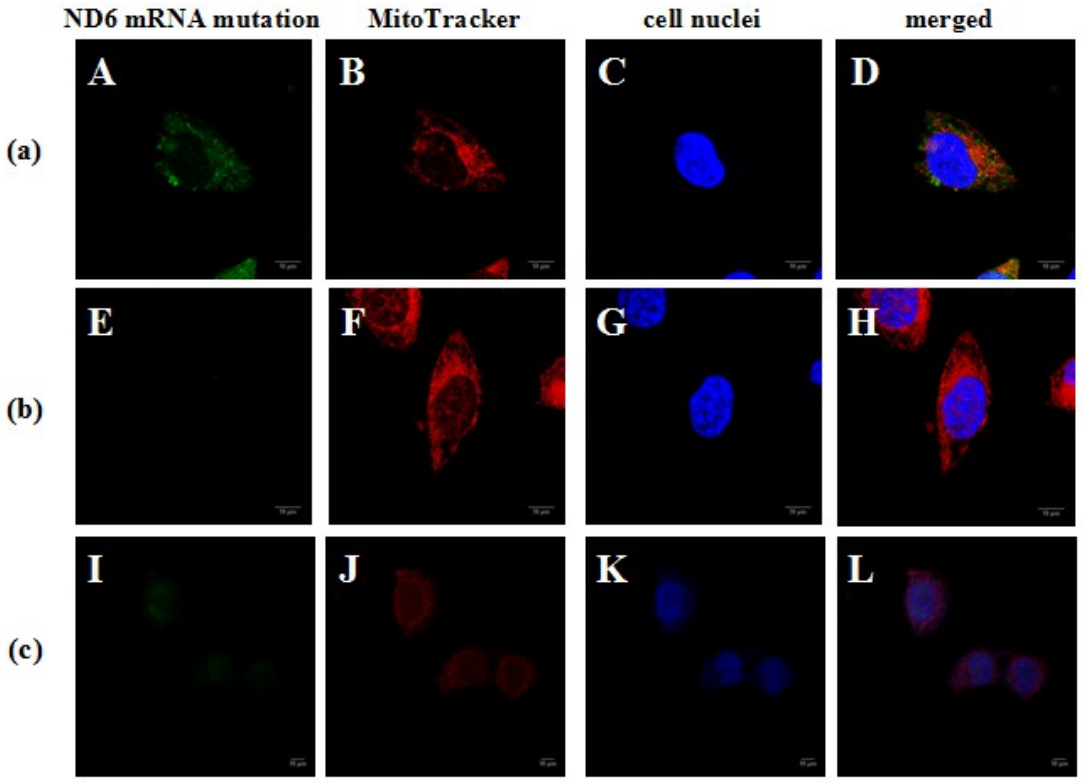

Figure S10. SFISH for ND6 mutation: SFISH-based fluorescence images of ND6 mRNA mutation in HepG2 cells (a), L02 cells (b), and HepG2 cells through random DNA instead of HP3 (c), respectively. The green fluorescence represents a FAM-labeled probes (A, E, I) for ND6 mRNA mutation, and the red fluorescence denotes Mito tracker (B, F, J) staining with mitochondria, and the blue fluorescence is DAPI (D, H, L) stained with nuclei.

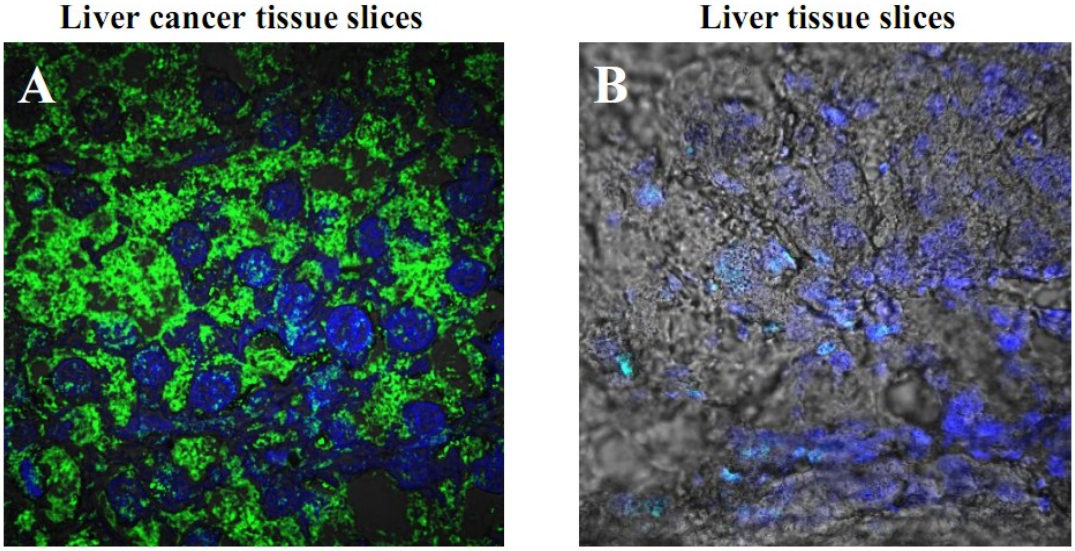

Figure S11. SFISH of TK1 mRNA in tissue slice: (A) liver cancer tissue slice; (B) Liver normal. The green fluorescence represents the FAM-labeled probes for TK1 mRNA, and the blue fluorescence is DAPI stained with cell nuclei.