A triphenylamine-based fluorescent probe with large Stokes shift for wash-free imaging of lipid droplets and diagnosis of fatty liver

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Experimental section

Measurements and apparatus

Chemicals for synthesis were purchased from Shanghai Aladdin Biochemical Technology Co.,Ltd, and used directly without further purification. The ^{1}H NMR spectroscopic measurements were conducted using a Bruker Advance 400 spectrometer. UV-Vis absorption spectra were measured on UV-5900 spectrophotometer. Fluorescence spectra were acquired using a HITACHI F-4600 fluorescence spectrophotometer (Measurement of parameters: λ_{ex}=450 nm; spectral slit width=10 nm; voltage=500 V). Cell imaging was performed on a Leica TCS SP8 confocal laser scanning microscope. Image data acquisition and processing were carried out utilizing Image J.

Synthesis of probe TSB

To a 100 mL round-bottomed flask, dissolve 5-(4-(diphenylamino)phenyl)thiophene-2-carbaldehyde (0.17 g, 0.5 mmol) completely in 15 mL of ethanol. Subsequently, add compound M2 (0.12 g, 0.5 mmol) and 80 μL piperidine into the flask. After refluxing for 24 hours, concentrate the organic layer and purify the crude product through silica gel column chromatography using a mixture of petroleum ether and ethyl acetate (50:1, V/V). Finally, isolate the balck

solid 0.09 g. ^{1}H NMR (400 MHz, DMSO) δ 8.36 (d, *J* = 15.3 Hz, 1H), 8.19-8.14 (m, 4H), 7.82-7.79 (m, 2H), 7.68-7.63 (m, 5H), 7.39-7.35 (m, 4H), 7.16-7.09 (m, 5H), 6.97 (d, *J* = 8.7 Hz, 2H), 6.81 (d, *J* = 15.3 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.61, 182.95, 146.94, 140.44, 137.73, 136.75, 135.45, 131.24, 129.52, 129.20, 129.02, 128.60, 127.08, 125.24, 123.97, 122.31, 117.79, 97.45, 29.70, 24.77. HRMS: Calculated 547.1589; found 547.1581.

TD-DFT Computation

The geometry of **TSB** at the ground state is optimized at the time-dependent density functional theory (TD-DFT)/ B3LYP functional level without any symmetry restraint using Gaussian 09 program. All theoretical calculation is obtained based on the optimized structures with B3LYP functional utilizing Gaussian 09 program package. The basis set of 6-31G* is chosen for all atoms. The molecular orbitals were visualized using GaussView 5.0.9.

Cytotoxicity assays

For cell viability studies, HepG2 cells were plated onto 96-well plates at a density of 10^4 cells per well. The cells were cultured until reaching approximately 90% confluence before being treated with **TSB** at specified concentrations in triplicate wells. Prior to compound treatment, fresh cell culture medium was substituted and aliquots from stock solutions of compounds were diluted accordingly to achieve desired final concentrations. After incubating for a duration of 24 hours under conditions maintained at a temperature of 37°C with CO_2 concentration set at around 5%, fresh DMEM medium was introduced as replacement for previous media content within each well . Subsequently, MTT solution having concentration equaling that of five milligrams per milliliter (5 mg/mL) was administered into every single well(10 μL/well). This mixture underwent further incubation lasting four more hours under same aforementioned conditions. Upon removing MTT-containing media, formazan crystals formed within each well would be dissolved by adding one hundred microliters (100 μL)of DMSO solvent . Finally, absorbance was measured at a

wavelength of 590 nm using a microplate reader (Infinite2000pro).

Confocal fluorescence imaging

The HepG2 cells were cultured on glass bottom plates (35 mm) at a density of 10^5 cells per plate. Before treatment, the cells reached approximately 65% confluence. To prepare **TSB** solution for experimentation, it was first dissolved in DMSO as stock solution (concentration: 1 mM), which was further diluted with DMEM cell culture medium to achieve the desired working concentration (10 μM). For live cell imaging analysis, HepG2 cells were incubated with **TSB** (10 μM) supplemented with cell medium containing FBS (10%) under controlled conditions: temperature maintained at 37 °C within an atmosphere composed of CO_2(5%) and air(95%), for a duration of 30 minutes. Following this incubation period, some samples underwent washing steps using PBS buffer before being subjected to imaging procedures; others skipped this step entirely. Cellular images were acquired using Leica TCS SP8 confocal laser scanning microscope while subsequent data processing utilized Image J software.

Co-localization experiments

Co-staining was performed using 1 μM BODIPY 493/503 and Lysotracker red for 30 min, respectively. For BODIPY, λ_{ex}= 488 nm; λ_{em}= 490-540 nm. For Lysotracker red, λ_{ex}= 561 nm; λ_{em}=580-630 nm. For **TSB**, λ_{ex}= 405 nm; λ_{em}= 520-580 nm.

Tissue and organ experiment

The procedures involving animals were approved by and adhered to the guidelines of Wannan Medical College. Efforts were made to minimize the number of animals used in these studies and measures were taken to reduce animal suffering from pain and discomfort. The NAFLD mice model was established by feeding C57 mice a 60% high-fat diet with choline-deficient feed for 8 weeks. In contrast, the control group mice were fed a normal diet for 8 weeks. Prior to imaging, probe **TSB**

(300 μM, 400 μL) was injected into the mice through their tail veins and allowed to circulate for 3 hours. Subsequently, both normal organs and fatty liver organs were isolated for organ imaging purposes. Additionally, sections measuring approximately 10 μm in thickness were obtained from a fraction of the liver tissue (including both normal liver tissue and fatty liver tissue) for further analysis via tissue imaging techniques. Before imaging, these sections underwent staining with **TSB** (at a concentration of 10 μM) at a temperature of 37°C for 1 h. Tissue imaging was performed using the Leica TCS SP8 system. Furthermore, H&E and Oil Red O staining experiments were conducted on the Olympus BX53 microscope.

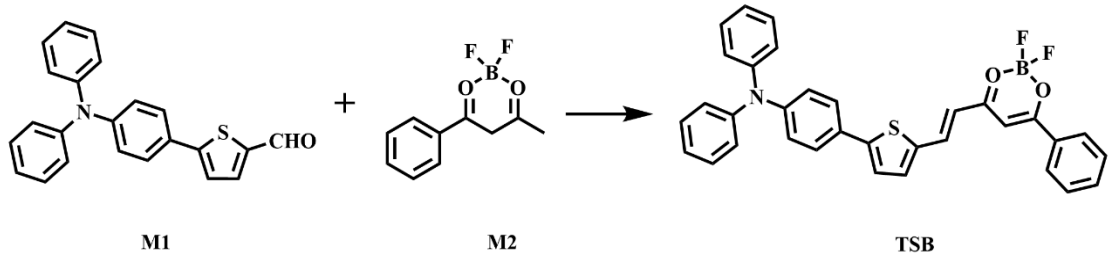

Figure S1 The synthetic route of **TSB**.

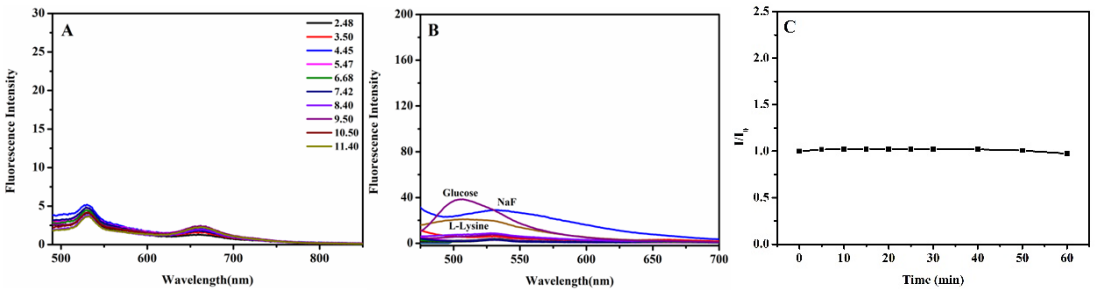

Figure S2 (A) Fluorescence spectra of **TSB** in different pH buffers; (B) Fluorescence spectra of **TSB** (10 μM) in the presence of different bioactive substances in PBS solution. The concentration of all analytes was 200 μM. 1, $Al_2(CO_3)_3$; 2, $CaBr_2$; 3, $CaCl_2$; 4, Captopril; 5, Cys; 6, DTT; 7, GSH; 8, H_2O_2; 9, L-Alanine; 10, L-Phenylalanine ; 11, L- Glutamic acid; 12, L- Arginine; 13, L- Lysine; 14, L- Proline; 15, L- Tryptophan; 16, L- Serine; 17, L- Threonine; 18, L- Valine; 19, L-Isoleucine; 20, L- Histidine; 21, $MgSO_4$; 22, Na_2S; 23, $Na_2S_2O_4$; 24, NaClO; 25, NaF; 26, $NaHCO_3$; 27, NaHS; 28, $NaNO_2$; 29, VC; 30, Glucose; 31, PBS. (C) Fluorescence

intensity ratio of **TSB** (10 μM) in DOA solvent over time.

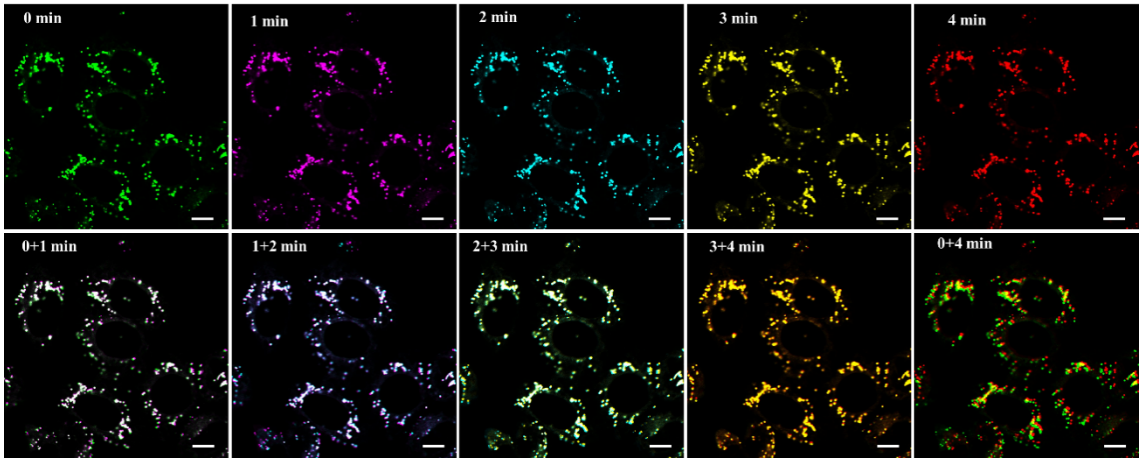

Figure S3 TSB (10 μM) staining is used to track the dynamic movement of LDs in HepG2 cells. Different pseudo-colors are used to display the movement of LDs at different time points (0, 1, 2, 3 and 4 min). Two images at different time points are overlapped and merged for better observing. (green represent 0 min, pink represent 1 min, cyan represent 2 min, yellow represent 3 min, red represent 4 min) Scale bar: 10 μm.

Table S1 The optical properties of **TSB**.

□	DOA	EA	THF	DMSO	EtOH	ACN	H_2O
$\lambda_{abs,\ max}$/nm	345	345	346	351	346	345	344
$\lambda_{em,\ max}$/nm	564	592	597	595	616	644	531
Stokes shift/nm	219	247	251	244	270	299	187
ε/L · moL^{-1} · cm^{-1}	23664	24102	23167	20480	25072	23464	18407

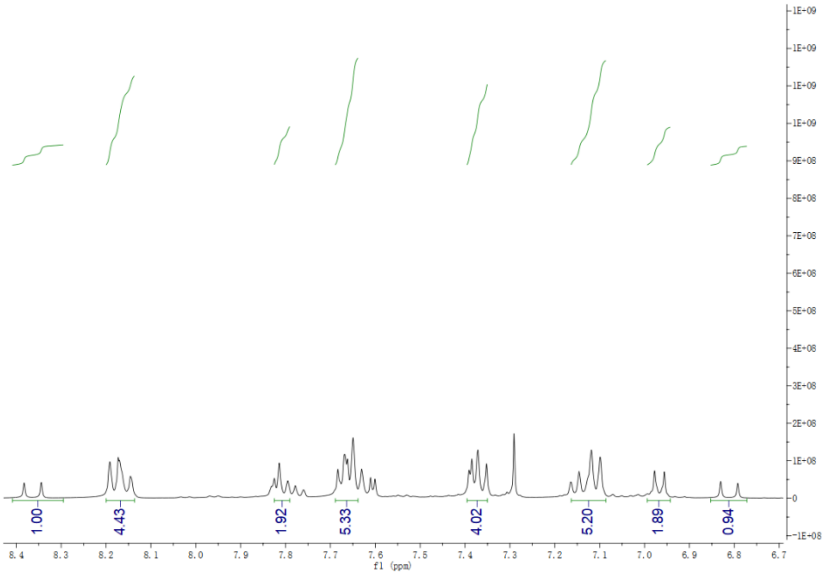

Figure S4 ^{1}H NMR spectra of probe **TSB**.

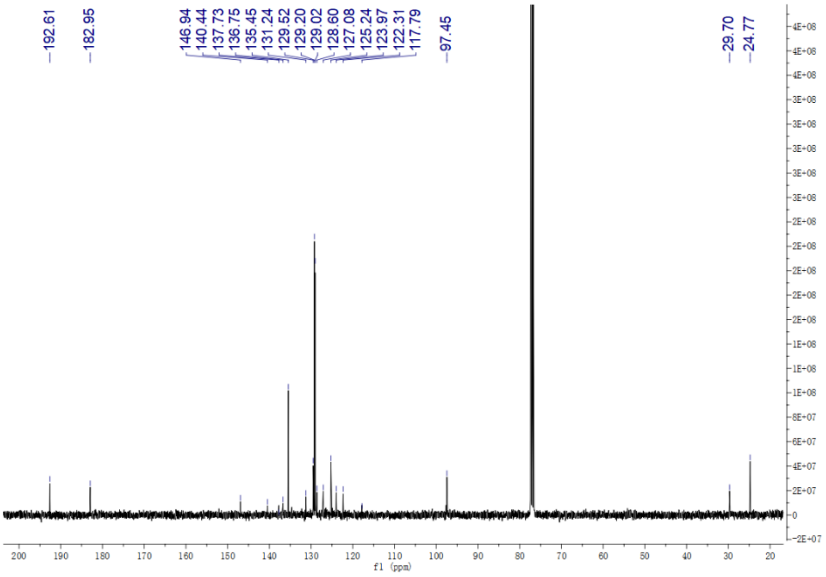

Figure S5 ^{13}C NMR spectra of probe **TSB**.

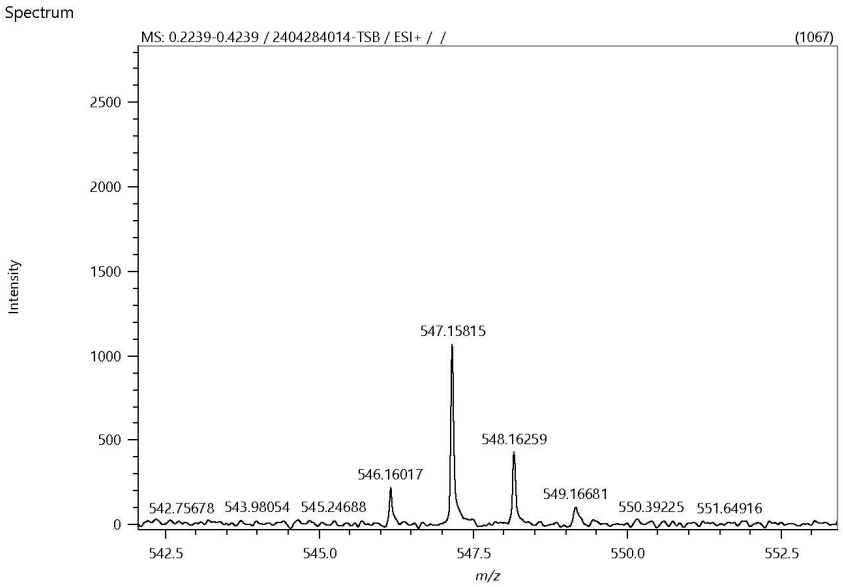

Figure S6 HRMS spectra of probe **TSB**.

Table S2 The comparison with the performance of known NAFLD-fluorescent probes.

Probe	Operating procedure	Stokes shift	Targeted	Application	Ref
TSB	Washing-free	299 nm	LDs	cell, tissues and organs imaging	this work
BDP-NIR-Py	Washing	26 nm	LDs	cell, tissues, organs and in vivo imaging	1
LIP-Ser	Washing	161 nm	LDs	cell, tissues and in vivo imaging	2
ISO-LD3	Washing-free	169 nm	LDs	cell, and tissues imaging	3
LD-1	Washing	118 nm	LDs	cell, organs and in vivo imaging	4
ZP-1	Washing-free	134 nm	LDs	cell, tissues, organs and in vivo imaging	5
CQ	Washing	205 nm	Mito	cell, organs and in vivo imaging	6
Er-V	Washing	100 nm	ER	cell, organs and in vivo imaging	7
CCB	Washing	169 nm	LDs	cell, and tissues imaging	8
lip-YB	Washing	181 nm	LDs	cell, and tissues imaging	9
PX-P	Washing	169 nm	LDs	cell, tissues and in vivo imaging	10

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