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Supplemental Information

Diagnosis of Fatty Liver Disease by a Multiphoton-Active and Lipid-Droplet-Specific AIEgen with Nonaromatic Rotors

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Materials and Methods

General

All chemicals were purchased from J&K Chemical Co. and Sigma-Aldrich and used as received without further purification. Anhydrous THF was used for fluorescence property investigation. Deionized water was used throughout this study. All aqueous solutions were freshly prepared with deionized water. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) and ^{19}F NMR (376 MHz) spectra were recorded on a Bruker ARX 400 spectrometer using tetramethylsilane (TMS) as internal standard. High-resolution mass spectra (HRMS) were recorded on a GCT premier CAB048 mass spectrometer operated in a MALDI-TOF mode.

One- and two-photon optical property measurements

Absorption and fluorescence spectra were recorded on Milton Roy Spectronic 3000 Array spectrometer and Perkin-Elmer LS 55 spectrofluometer, respectively using quartz cuvettes with a path length of 1 cm. All absolute fluorescence quantum yields were determined using an integrating sphere. Two-photon (TP) excited fluorescence (TPEF) spectra of ABCXF in THF were measured on a Coherent Mira 900 and the pump laser beam (800–1000 nm) came from a mode-locked Ti:sapphire laser system at the pulse duration of femtosecond pulses with a repetition rate of 76 MHz. Two-photon absorption cross sections were measured using the two-photon-induced fluorescence method with rhodamine B in methanol as the standard[1]. The TP cross sections of ABCXF were calculated according to the equation: $\delta_x = \delta_{st}[F_x\Phi_{st}\eta_{st}c_{st}]/[F_{st}\Phi_x\eta_x c_x]$, where δ was the TPA cross section, F is the integrated intensity of two-photon excited fluorescence, Φ is the fluorescence quantum yield, η is the overall fluorescence collection efficiency of the experimental apparatus, and c is the concentration of the solution. The subscripts x and st refer to the unknown samples and standard material, respectively.

Cell cultures and live cell imaging

HeLa and HepG2 cells were cultured and regularly passaged in the culture medium (Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin) at 5% CO_2/air at 37 ℃ in a humidified incubator. A day prior to the experiment, the cells were split, and approximately $8*10^5$ cells were seeded onto each coverslip in 35 mm petri dish. HeLa and Hep G2 cells were incubated in fresh DMEM medium containing 200 nM of ABCXF or Nile Red (1 mM ABCXF and Nile Red stock solutions were prepared in DMSO) at 37 ℃ in 5% CO_2 for 20 min before live cell imaging. For co-stain imaging, HeLa and Hep G2 cells were incubated with 2 μM of TTV (1 mM TTV stock solution was prepared in DMSO). After incubation, the cells were washed three times with phosphate-buffered saline (PBS, pH = 7.4). One-photon

confocal fluorescence imaging was obtained on Zeiss LSM 800 confocal scanning microscope (excitation = 488 nm and emission collection: 500-600 nm for ABCXF; excitation = 405 nm and emission collection: 420-500 nm for TTV; excitation = 488 nm and emission collection: 540-700 nm for Nile Red). For the photostability test, the cells containing different dyes were continuously irradiated with confocal laser for 20 min (excitation = 488 nm for ABCXF, BODIPY 493/503 and Nile Red, laser power = 4 %).

Animals and treatment

30 male Hartley guinea pigs (300±30g) were purchased from Guangdong Experimental Animal Center (Foshan, China). The animal experiment was completed at the Experimental Animal Center of Guangzhou University of Chinese Medicine (SYXK (Yue) 2018-0085). All guinea pigs were kept at constant temperature (20-25 °C) and 65-70% humidity with a 12-hour light/dark cycle in independent ventilation cage. Animals were free to drink deionized water and feed the corresponding regular or high fat feed (provided by Beijing Botaihongda Biotechnology Co., Ltd.). All guinea pigs were randomly divided into a blank group (5 guinea pigs) and a model group (5 guinea pigs) after one week of adaptive feeding. The blank group was fed with normal feed, and the model group was fed with high-fat feed. After feeding for five weeks, the guinea pigs were anesthetized with 10% chloral hydrate (0.3 mL/100g), and the livers were taken.[2] A part of the liver tissue was used for sectioning and Oil red O staining, and the remaining liver tissue was stored at -80 °C. These experiments were performed in compliance with the relevant laws and institutional guidelines. The institutional committee had approved the experiments.

Oil Red O staining

The frozen liver tissue was embedded with OCT agent in a cryostat (Thermo Scientific, USA) and sectioned with different thickness. The frozen sections (10 μm) were infiltrated with distilled water, stained with Oil Red O staining solution for 10 min, and then rinsed by 60% isopropyl alcohol for 1-2 min. After these, hematoxylin solution was used to stain the sections for 30-60 s. Subsequently, it was washed with running water for 2 min. After the water around the slice was blotted dry, it was sealed with glycerin gelatin. Finally, the film was observed with an optical microscope (Olympus, Japan).[3]

Liver tissue imaging

The liver tissues with the thickness of 10 μm, were stained with the culture medium containing ABCXF (1 μM) or Nile Red (1 μM) in 5% CO_2/air humidified incubator at 37 ℃ for 1 h. The tissues were washed three times with PBS (pH = 7.4) prior to one- and two-photon imaging. For one-photon imaging, same experimental condition was used as live cell imaging. Two-photon fluorescence imaging was performed on Leica DMI 6000 STED super-resolution confocal laser scanning

microscope. For two-photon experiments, the excitation wavelength of 850 nm from a Ti:sapphire femtosecond laser source (coherent chamelon ultra) was used and the incident power on samples was modified by means of an attenuator and examined with a power monitor (Coherent). A multiphoton emission filter (FF01–750; Semrock) was used to block the IR laser. Same procedure was followed for the tissues with the thickness of 100 μm, with DMEM containing ABCXF (2 μM). For the photostability test, tissue samples stained with ABCXF, BODIPY 493/503 and Nile Red (2 μM) were continuously irradiated at wavelength of 850 nm from a Ti:sapphire femtosecond laser source for 10 min.

Cell viability assay

The cytotoxicity of ABCXF was investigated by the standard MTT assay. Around 5000 cells were seeded into each well of 96-well plate and allowed to adhere for 24 h. 200 μL of culture medium containing of ABCXF was added into each well (concentration range: 0.5 ~ 10 μM) and DMEM with only DMSO was added into wells to serve as a negative control group. The cells were incubated for 24 h at 5% CO_2/air at 37 ℃ in a humidified incubator. MTT solution (5 mg/mL in DMEM) was added into each well and cells were incubated for another 4 h. The MTT solution was replaced with 100 μL of DMSO for each well to lysis cells and dissolve the purple crystal. 96-well plate was shaken on the shaker for 5 min and the optical density (OD) readings at 570 nm were taken using a plate reader.

Synthetic details

Synthesis of ABCXF. Compound **2** (253 mg, 1.0 mmol) and *t*-BuONa (153 mg, 1.6 mmol) were added to anhydrous EtOH (10 ml) in a round bottomed flask and stirred for 5 minutes at the room temperature. Then compound **1** (210 mg, 1.2 mmol) was added and the mixture was refluxed at 90 ℃ for 5 h. After cooling down to the room temperature, all the solvent was removed under the reduced pressure. The residue was purified by silica chromatography using Hexane/Chloroform mixture (from 5:1 to 1:1, *v*/*v*) as an eluent to give ABCXF as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.98 (s, 2H), 7.78 (s, 1H), 7.53 (d, J = 10.9 Hz, 1H), 7.48 (d, J = 8.7 Hz, 2H), 7.25–7.06 (m, 2H), 6.69 (d, J = 8.7 Hz, 2H), 3.06 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 151.85, 145.93, 145.49, 136.37, 132.48 (q, J = 33.6 Hz), 129.89, 124.88 (d, J = 2.7 Hz), 124.44, 123.15, 121.73, 121.30 (m), 119.72, 116.79, 111.96, 105.59, 40.13. ^{19}F NMR (376 MHz, $CDCl_3$), δ (ppm): -63.00 (s, 6F). HRMS (MALDI-TOF): m/z calcd for $[C_{21}H_{16}F_6N_2]$ 410.1218, found 410.1211 ([M]).

NMR spectra

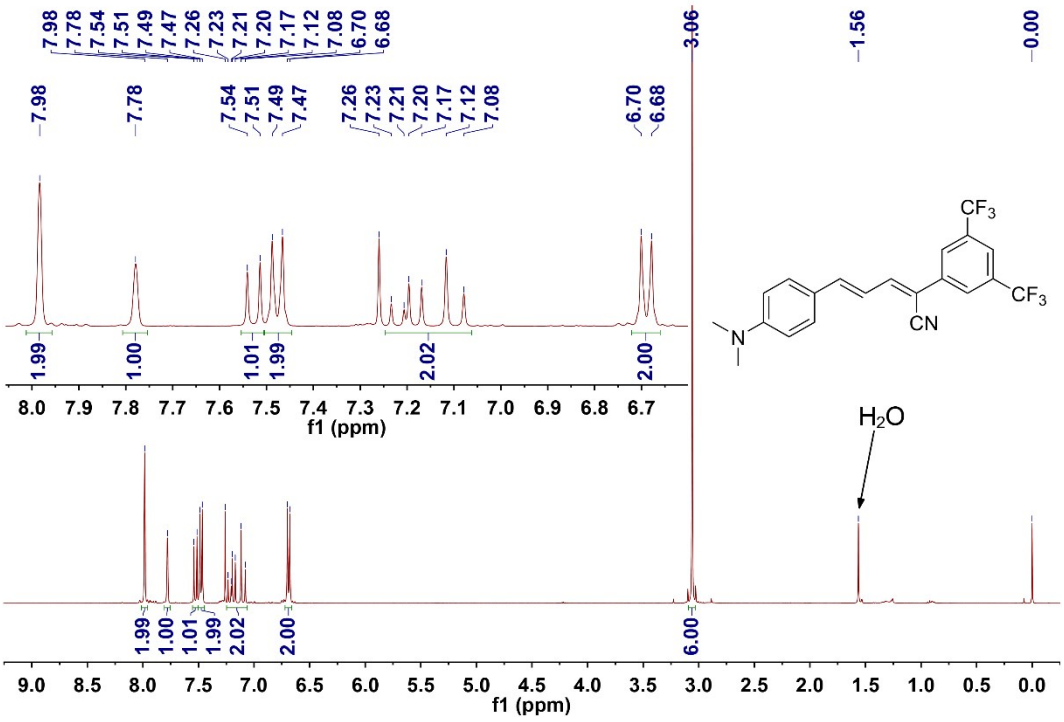

Fig. S1. ^{1}H NMR spectrum of ABCXF in $CDCl_3$

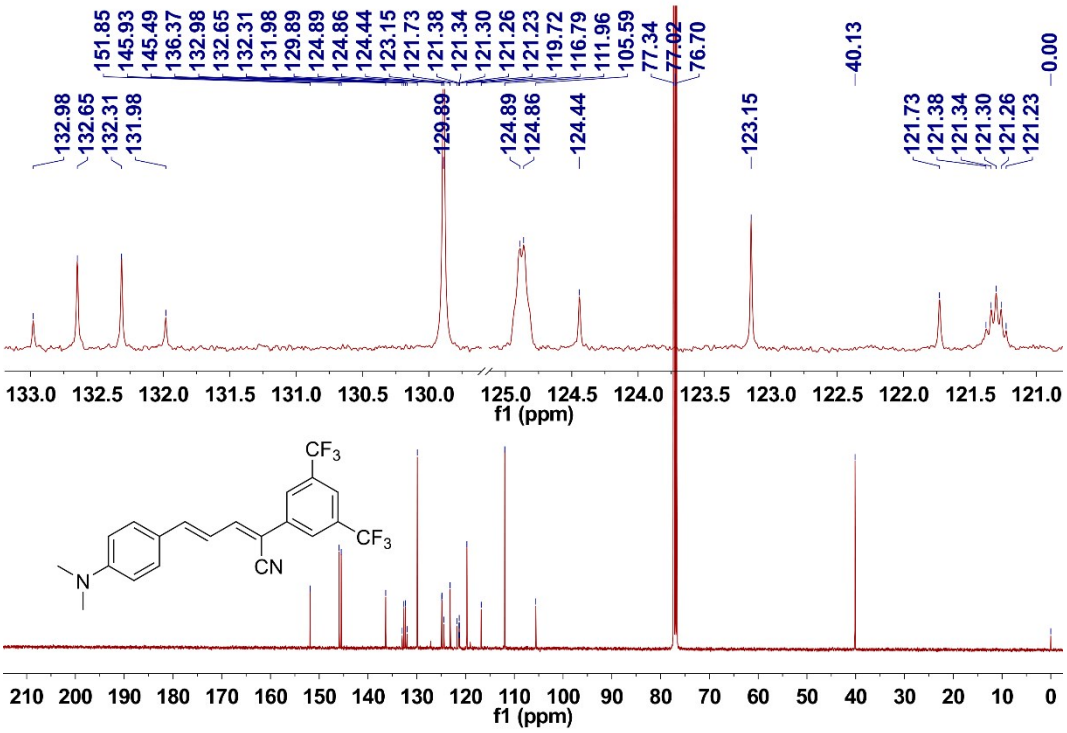

Fig. S2. ^{13}C NMR spectrum of ABCXF in $CDCl_3$

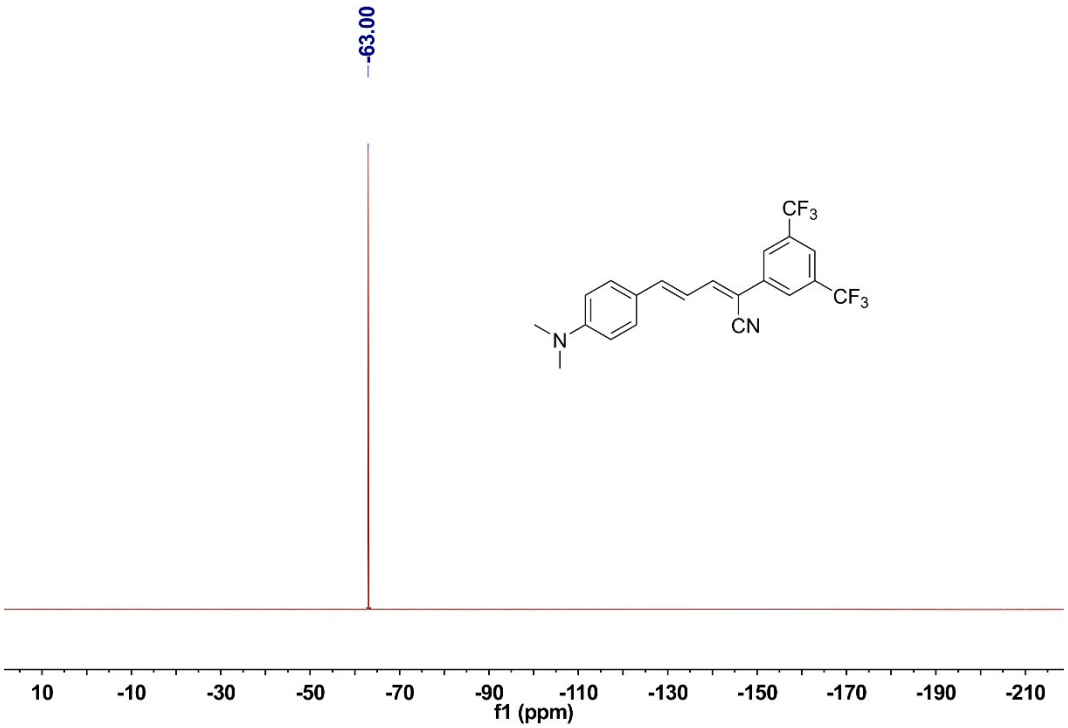

Fig. S3. ^{19}F NMR spectrum of ABCXF in $CDCl_3$

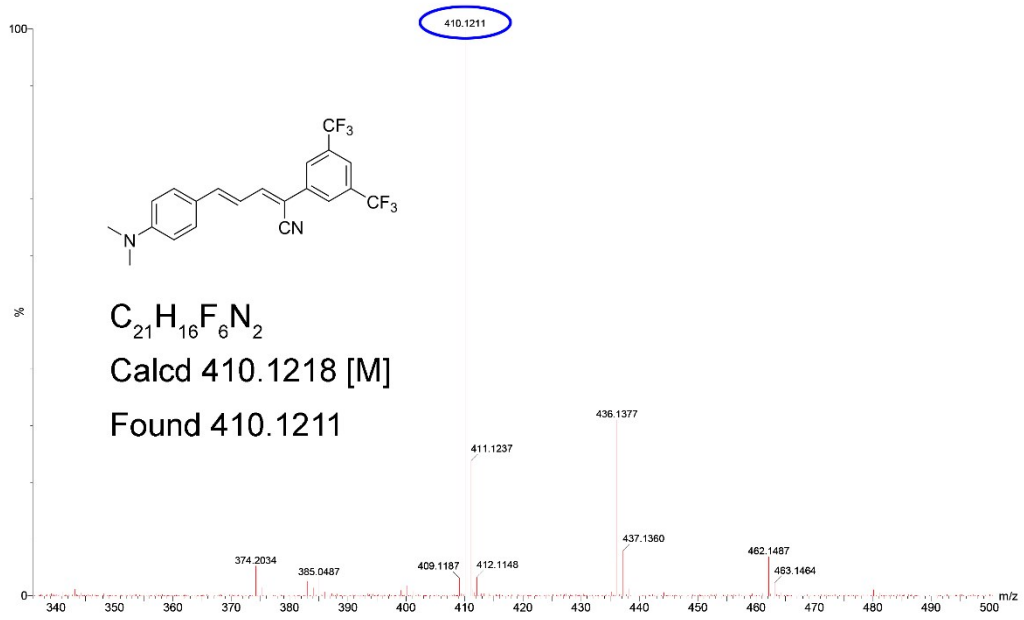

Fig. S4. High-resolution mass spectrum of ABCXF.

Crystallographic data

Table S1. Crystal data and structure refinement for ABCXF.

Bond precision:	C-C = 0.0022 A		Wavelength=1.54178
Cell:	a=8.5369(6)	b=9.6061(7)	c=12.6366(8)
	alpha=69.429(3)	beta=88.085(4)	gamma=84.076(3)
Temperature: 271 K			
	Calculated		Reported
Volume	965.02(12)		965.02(12)
Space group	P -1		P -1
Hall group	-P 1		-P 1
Moiety formula	C21 H16 F6 N2		C21 H16 F6 N2
Sum formula	C21 H16 F6 N2		C21 H16 F6 N2
Mr	410.36		410.36
Dx,g cm-3	1.412		1.412
Z	2		2
Mu (mm-1)	1.084		1.084
F000	420.0		420.0
F000'	421.69		
h,k,lmax	10,11,14		10,11,14
Nref	3281		3154
Tmin,Tmax	0.771,0.850		0.521,0.852
Tmin'	0.582		
Correction method= # Reported T Limits: Tmin=0.521 Tmax=0.852 AbsCorr = MULTI-SCAN			
Data completeness= 0.961		Theta(max)= 64.998	
R(reflections)= 0.0504(2502)		wR2(reflections)= 0.1464(3154)	
S = 1.100	Npar= 320		

Photophysical data

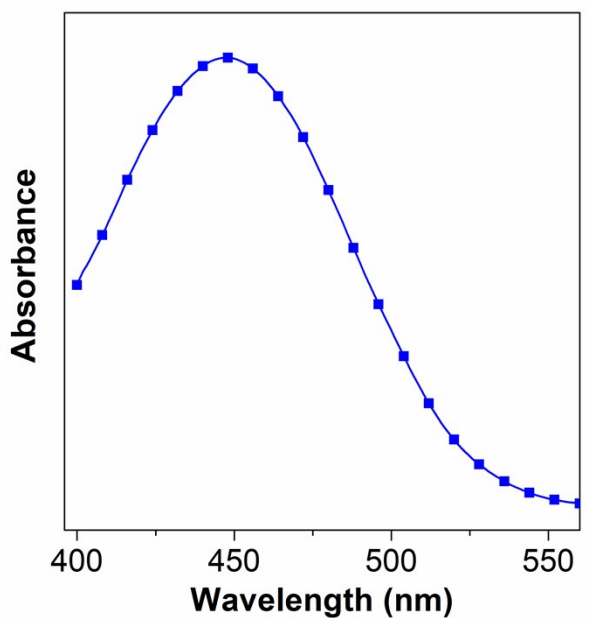

Fig. S5. Absorption spectra of ABCXF (10 μM) in THF.

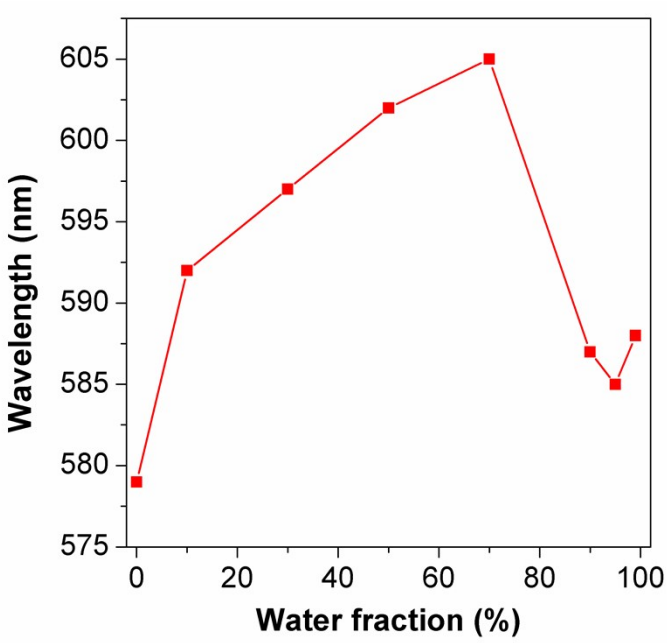

Fig. S6. The plot of the maximum emission wavelength versus the composition of THF/water mixture containing ABCXF (10 μM).

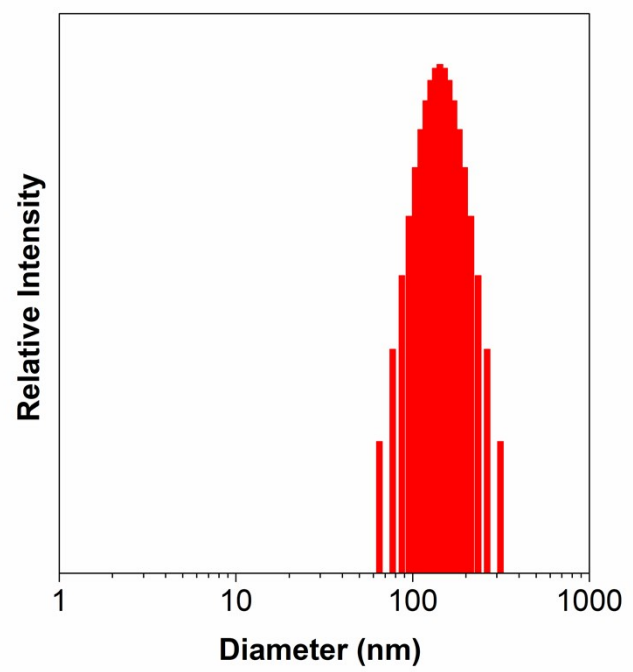

Fig. S7. Dynamic light scattering data of ABCXF in water containing 1% of THF. Hydrated diameter: 142 nm.

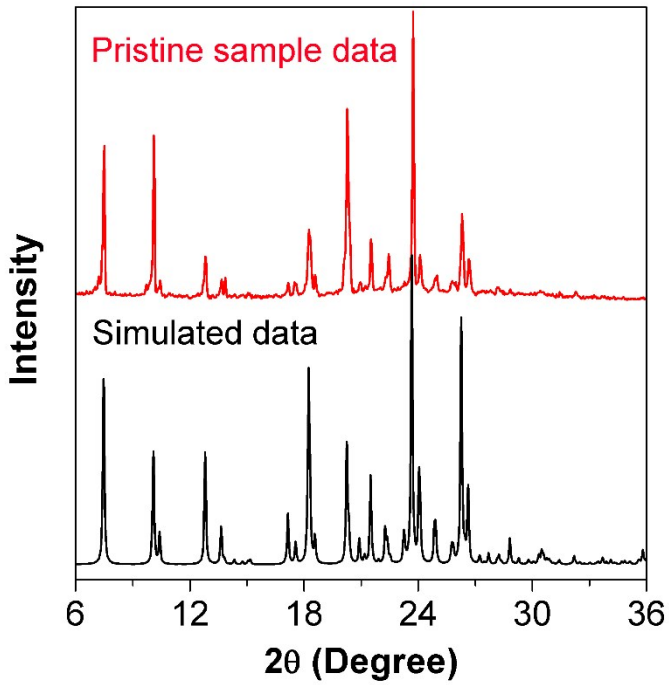

Fig. S8. XRD pattern of the pristine sample and simulated XRD pattern of ABCXF.

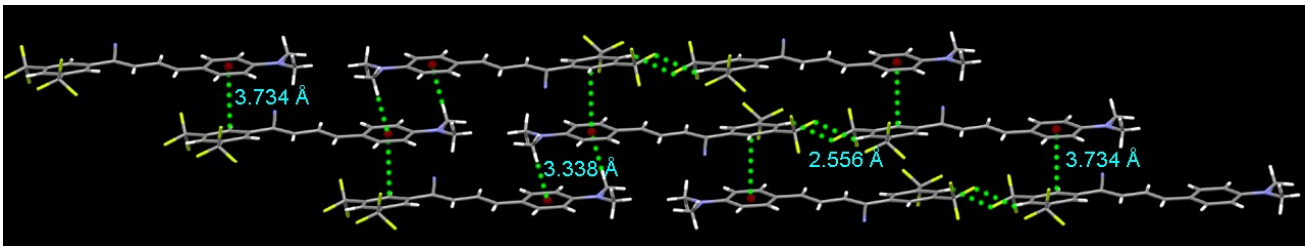

Figure S9. The principal intermolecular packing interactions of ABCXF.

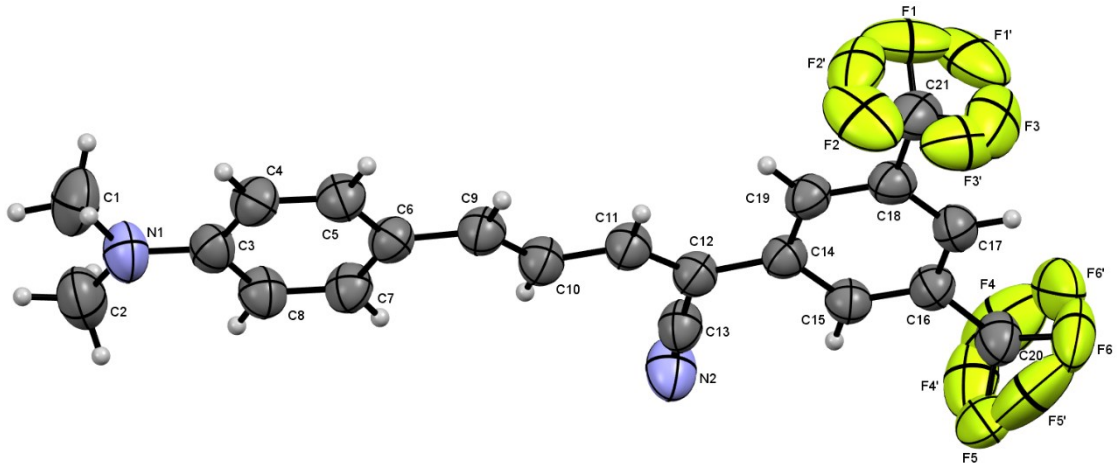

Fig. S10. Single crystal structure of ABCXF with disordered CF_3 groups. All H atoms are omitted for clarity

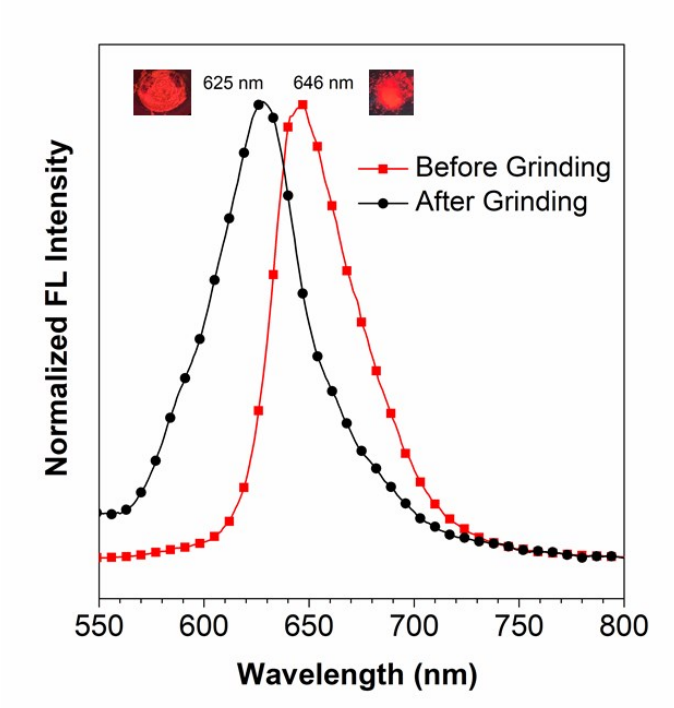

Fig. S11. Change in the emission spectrum of ABCXF after grinding process.

Table S2. Quantum yield (%) of ABCXF (10 μM) dissolved in different solvents.

Solvent	QY (%)
DMSO	2.1
DMF	1.2
Acetonitrile	0.8
Acetone	0.9
EtOH	0.5
MeOH	0.7
THF	0.8
DCM	0.5
Chloroform	0.6
Ethyl ether	0.4
Toluene	0.3

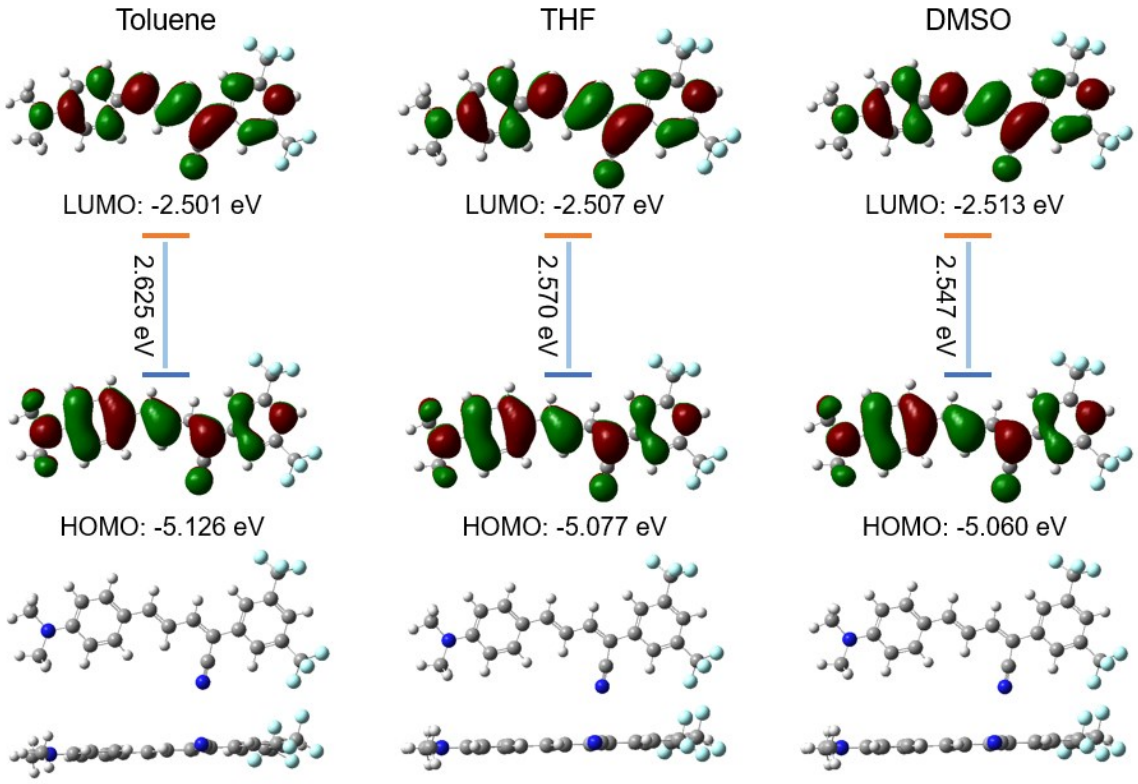

Fig. S12. Frontier molecular orbitals of ABCXF in toluene, THF and DMSO, at excited state calculated by B3LYP/6-31G(d, p) method, Gaussian 09 program.

Table S3. Energy gap and oscillator strength in Toluene, THF and DMSO, at excited state calculated by B3LYP/6-31G(d, p) method, Gaussian 09 program.

S_1	Toluene	THF	DMSO
Energy gap (eV)	2.461	2.287	2.203
Oscillator strength	1.6848	1.8238	1.8796

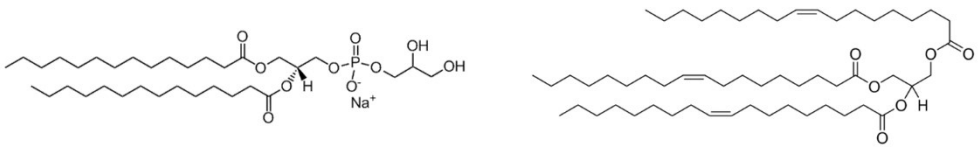

Fig. S13. Chemical structures of DMPG and TAG.

Imaging data

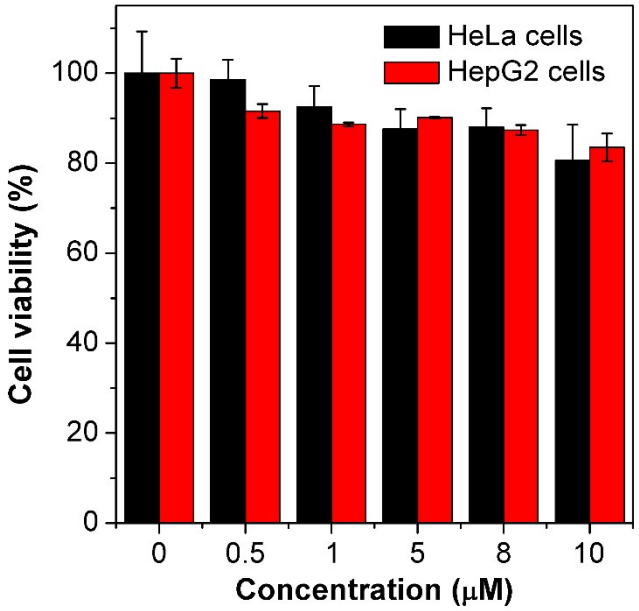

Fig. S14. Cell viability of HeLa and HepG2 cells stained with different concentrations of ABCXF for 24 h.

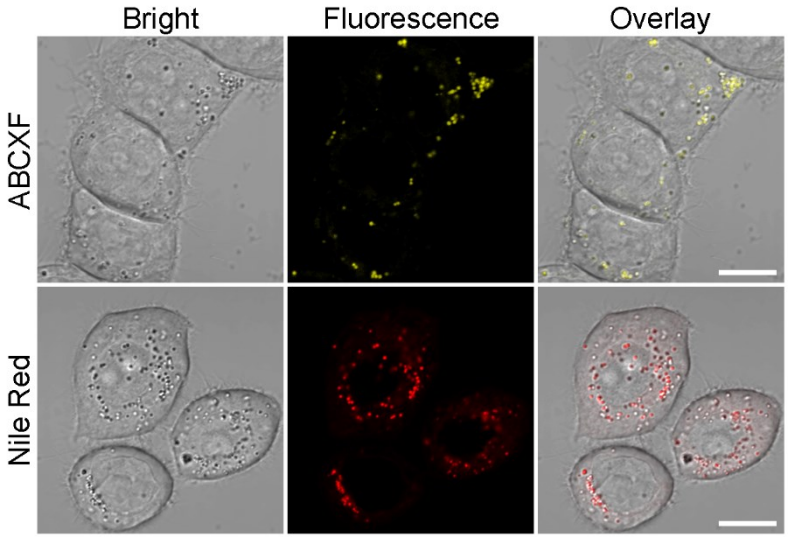

Fig. S15. Confocal laser scanning microscopy (λ_{ex} = 488 nm) images of HepG2 cells stained with ABCXF (200 nM) and Nile Red (200 nM). Scale bar: 10 μm.

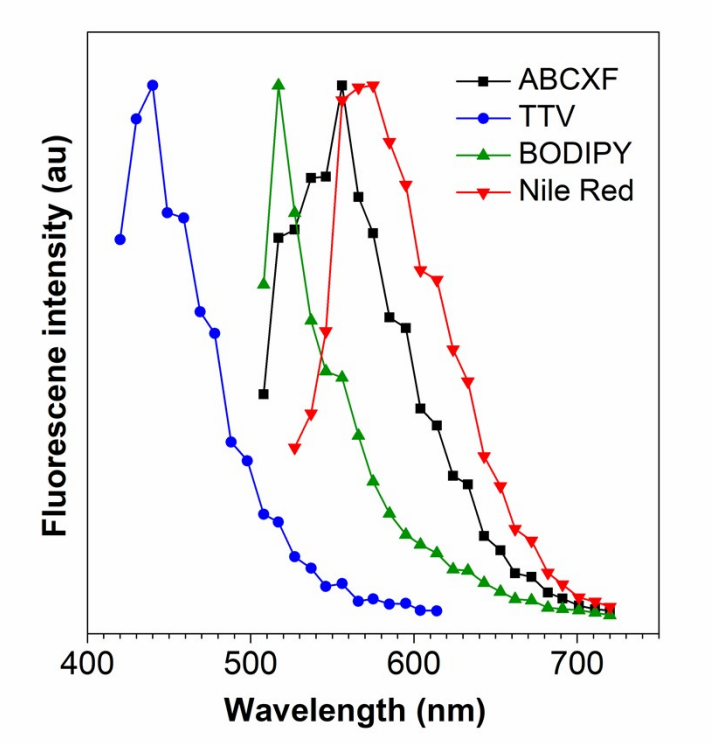

Fig. S16. *In situ* fluorescence spectra of ABCXF, TTV, BODIPY 493/503 and Nile Red in HeLa cells (λ_{ex} = 405 nm for TTV; λ_{ex} = 488 nm for ABCXF, BODIPY 492/503 and Nile Red).

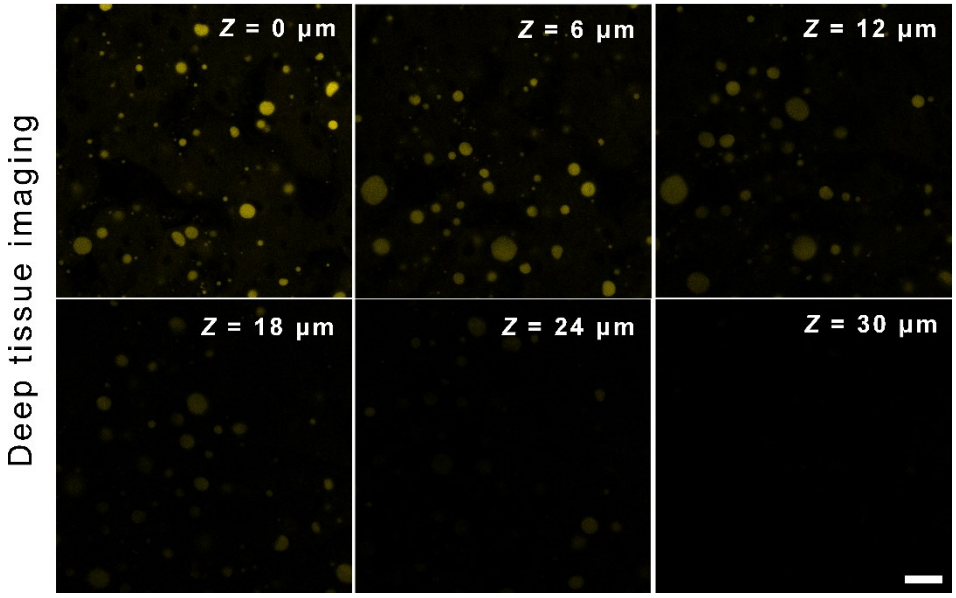

Fig. S17. *Ex vivo* fatty liver tissue imaging. One-photon (λ_{ex} = 488 nm) images of high-fat feeding mice liver tissue stained with ABCXF (2 μM) at different penetration depths. Scale bar: 20 μm.

References

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2. G. Barja, M. Lopez-Torres, R. Perez-Campo, C. Rojas, S. Cadenas, J. Prat and R. Pamplona, Dietary vitamin C decreases endogenous protein oxidative damage, malondialdehyde, and lipid peroxidation and maintains fatty acid unsaturation in the guinea pig liver, *Free Radic Biol Med*, 1994, **17**, 105-115.
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