Electronic Supplementary Material (ESI) for New Journal of Chemistry.
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A molecular probe based on pyrimidine imidazole derivatives for stable super-resolution endoplasmic reticulum imaging in living cells

Ling Hu [a‡], Sajid Hussain[a‡], TianyanLiu[a], YuanzhenYue[a], JiejieLiu[b], XiaoheTian*[a], YupengTian[b]

[a]School of Life Science, Anhui University, Hefei 230601, P.R. China

[b]Department of Chemistry, Key Laboratory of Functional Inorganic Material Chemistry of Anhui Province, Anhui University, Hefei 230601, P. R. China

‡These authors contributed equally to this work.

E-mail address: xiaohe.t@ahu.edu.cn

Experimental section

Synthesis and characterization

The synthetic route for the chromophore (**EX-1**) is illustrated in **Scheme 1**. Through the Solvent-free Wittig reaction, **EX-1** was obtained in high yields. The structure ofchromophore **EX-1** was characterized by IR, ^{1}H NMR, ^{13}C NMR spectra and elemental analyses. Additionally, **EX-1**was further confirmed by single crystal X-ray diffraction analysis.

Materials and Apparatus

All chemicals and solvents were dried and purified by usual methods. IR spectra (4000–400 cm^{-1}), as KBr pellets, were recorded on a Nicolet FT–IR 170 SX spectrophotometer. Mass spectra were obtained on a Micromass GCT-MS Spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 400 spectrometer with tms as an internal standard.

Optical Measurements

The 1PA spectra were measured on a UV-3600 spectrophotometer. The 1PEF measurements were performed by using an F-2500 fluorescence spectrophotometer. The concentration of the sample solution was 1.0×10^{-5}mol/L. The fluorescence quantum yields (Φ) were determined by using coumarin 307 as the reference. Quantum yields were corrected as follows:

$$\Phi_s = \Phi_r\left(\frac{A_r n_s^2 D_s}{A_s n_r^2 D_r}\right)$$

Where the *s* and*r* indices designate the sample and reference samples, respectively, *A* is the absorbance at λ_{exc}, η is the average refractive index of the appropriate solution, and *D* is the integrated area under the corrected emission spectrum. For time-resolved fluorescence measurements, the fluorescence signals were collimated and focused onto the entrance slit of a monochromator with the output plane equipped with a photomultiplier tube (HORIBA HuoroMax-4P). The decays were analyzed by 'least-squares'. The quality of the exponential fits was evaluated by the goodness of fit (χ^2).

Two-Photon Excited Fluorescence (2PEF) Spectroscopy and Two-Photon Absorption (2PA) Cross-Section

2PA cross-sections (δ) of the samples were obtained by the two-photon excited fluorescence (2PEF) method with a femtosecond laser pulse and a Ti:sapphire system (700–920 nm, 80 MHz, 140 fs) as the light source. The concentration of the sample solution was 1.0×10^{-3} M. Thus, the δ values of samples were determined by the following Equation (1). $\delta_s=\delta_r\cdot F_s\cdot \Phi_r\cdot C_r\cdot n_r/F_r\cdot \Phi_s\cdot C_s\cdot n_s$ where the subscripts "s" and "r" represent sample and reference (here, fluorescein in ethanol solution at a concentration of 1.0×10^{-3} mol/L was used as reference), respectively. F is the overall fluorescence collection efficiency intensity of the fluorescence signal collected by the fiber spectra meter. Φ, *n,*and*c* are the quantum yield of the fluorescence, the refractive index of the solvent, and the concentration of the solution, respectively.

As shown in **Figures S2**, there is no linear absorption in the wavelength range 600-900 nm for all the compounds in DMF, which indicates that there are no energy levels corresponding to an electron transition in this spectral range. The Log-Log linear of the squared dependence of induced fluorescence signal and incident irradiance intensity of EX-1 was 2.02, which should be safely attributed to multiphoton absorption excited fluorescence (**Figure S3**). The details of determination conditions are given in the Experimental Section. Detailed experiments revealed that the peak positions of the 2PEF spectra of this chromophore are independent of the excitation wavelengths, but the emission intensities of the 2PEF are dependent on the excitation wavelengths. The electrons can be pumped to the different excited states by 2PA due to the different selection rules, but they would finally relax to the same lowest excited state *via* internal conversion and/or

vibrational relaxation.

2-imidazolyl-4-methyl-pyrimidine (**M1**)

CuI (0.19 g, 1 mmol), 1,10-phenanthroline (0.6 g, 3 mmol) and DMF (5 mL) were added to a three-necked flask equipped with a magnetic stirrer and a reflux condenser. The reaction mixture turned brown and was kept stirring for 5 min at room temperature, then *t*-BuOK (1.12 g, 10 mmol), pyrazole (0.68 g, 10 mmol), 2-iodide-4-methyl-pyrimidine(0.44 g, 2 mmol), and catalytic amount of 18-crown-6 were added orderly. After complete addition, the mixture was heated at reflux under nitrogen for about 2hand cooled to room temperature. The residue was diluted with 200 mL of dichloromethane, washed four times with distilled water, and dried with anhydrous $MgSO_4$. Then it was filtered and concentrated, purified by flash column chromatography on silica. Elution with petroleum/ethyl acetate (6:1 v/v) gave red solid **M1** 0.39 g, Yield: 80 %.^{1}H-NMR: (400 MHz, CD_3COCD_3), δ (ppm): 8.523 (s, 1H), 7.185 (s,1H), 7.920 (s, 1H), 7.088 (s, 1H), 2.515 (s, 6H)。M^+ (MS/ESI), 161.31.

(E)-4-(2-(2-(1H-imidazol-1-yl)pyrimidin-4-yl)vinyl)-N,N-diphenylaniline (**EX-1**)

t-BuOK (0.56 g, 5 mmol), 4-(dibutylamino)benzaldehyde (2.79 g (12 mmol)) and M1 (1.92 g (12 mmol)) were mixed together and milled vigorously for about 10 min. The reaction was monitored by TLC (petroleum/ethyl acetate 4:1 v/v). After the reaction was completed, the mixture was dispersed in 200 mL of distilled water. The solution was extracted with CH_2Cl_2 several times. The organic layer was washed with water, saturated brine and dried over anhydrous $MgSO_4$. After removing solvent under reduced pressure, the residue was purified by flash chromatography on silica gel using petroleum/ethyl acetate (8:1 v/v) as eluent and gave yellow micro-crystals **EX-1** 0.25 g, yield: 65 %。^{1}H-NMR (CD_3COCD_3, 400MHz) δ= 0.96~0.99 (t,J = 6.00Hz, 6H), δ= 1.35-1.42 (m, 4H),1.56~1.64(m, 4H),δ = 3.30~3.34 (t,J = 8.00Hz, 4H), δ =6.63~6.65 (d, J=8.00 Hz,4H), δ =6.67~6.81 (d, J=16.00Hz, 2H), δ =7.04~7.06 (d, J=8.00Hz, 1H), δ =7.19 (s, 1H), δ =7.47~7.49 (d, J=8.00Hz, 2H), δ =7.85~7.89 (d, J=16.00Hz, 4H), δ=7.96 (s, 1H), δ=8.46~8.48 (d, J=8.00Hz, 2H), δ=8.71 (s, 1H). ^{13}C-NMR (CD_3COCD_3, 100 MHz) 15.82, 22.40, 31.11, 59.98, 113.73, 116.67, 120.46, 123.55, 126.31, 128.13, 131.22, 134.54, 147.18, 159.11, 165.92, 167.10. MALDI-TOF, m/z(%):374. 43 ([M^+, 100). m.p.102 ^{o}C.

Cell culture

HepG2 cells were seeded in 24-well glass bottom plates (Corning®, P2415HN) with the density of $1X10^4$ cells per well and cultured for 96 h. For live cell imaging, the culture medium DMEM was supplemented with 10 % FCS, streptomycin and penicillin, fungizone and L-glutamine at 37 ^oC under 5 % CO_2 and 95 % air for 30 min incubation. PBS (2x2 ml per well) wash was given to the cells and 2ml of PBS was added to each well. The cells were imaged using confocal laser scanning microscopy.

Cell cytotoxicity

The cytotoxicity of the **EX-1** compound on HepG2 cells was monitored by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay by incubating with a concentration range of compounds from 1μM to 50 μM. Cells were trypsinized and plated to 70 % confluence in 96 well plates 24 h before treatment. All compounds were then added at indicated concentrations to triplicate wells. Prior to the compounds' treatment, the DMEM was removed and replaced with fresh DMEM, and aliquots of the compounds stock solutions were diluted to obtain the final concentrations of 1, 5, 10, 25 and 50μM. The treated cells were incubated for 24 h at 37 ℃ and under 5 % CO_2. Subsequently, the cells were treated with 5 mg/mL MTT (10μL per well) and incubated for an additional 4 h (37 ℃, 5 % CO_2). Then, DMEM was removed; the formazan crystals were dissolved in DMSO (100 μL per well), and the absorbance at 490 nm using a microplate reader (SpectraMax Paradigm).

Cell imaging using confocal laser scanning microscopy

The images of the cells were taken on Leica SP8 confocal laser scanning microscope with 100x oil lens. A Coherent chameleon pulsed infrared multi-photon laser was used for two-photon imaging (at wavelength; 900 nm) check. For real-time live cell imaging, an incubation chamber was connected to the ZEISS temperature control unit 37 ^oC and CO_2 controller with proper humidity. For complex **EX-1**, the excitation wavelength of 820 nm was used and the emission was measured at 560-620 nm wavelength ranges. Costaining was performed by incubating cells with 10μM ERtracker-Red (E_x = 579 nm, E_m = 585-610 nm) and 10 μM **EX-1** for 30 min. To obtain and

process imaging data, Leica SP8 and Image J were used.

Stimulated emission depletion (STED) nanoscopy

STED nanoscopy experiments were performed under Leica DMi8 confocal microscopy equipped with Leica TCS SP8 STED-ONE unit and the compound was excited under STED laser, the emission signals were collected using HyD reflected light detectors (RLDs). Specimen living cells were prepared using a similar method as normal confocal microscopy described previously, and donut laser used in 595 nm STED laser (70 % power), with 2048*2048 pixel and *100 scanning speed. The STED micrographs were further processed 'deconvolution wizard' function using Huygens Professional software (version: 16.05) under authorized license. The area radiuses were estimated under 0.02 micros with the exclusion of 100 absolute background values. Maximum iterations were 40-time, signal-to-noise ration 20 was applied, with quality threshold 0.05; iteration mode: Optimized; Brick layout: Auto.

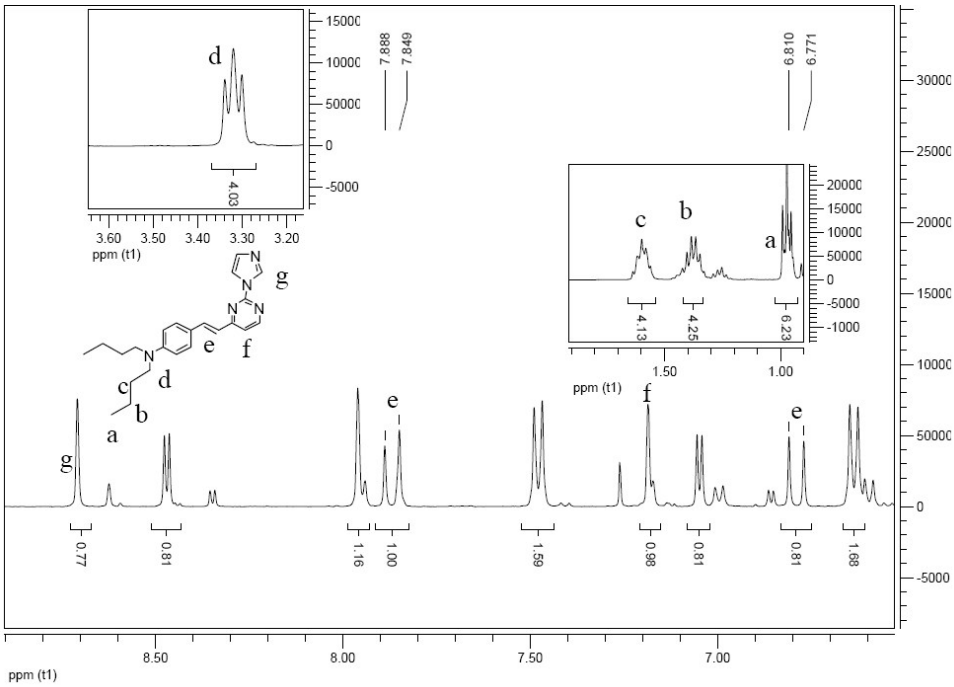

Figure S1.^{1}H-NMR spectrum of **EX-1**

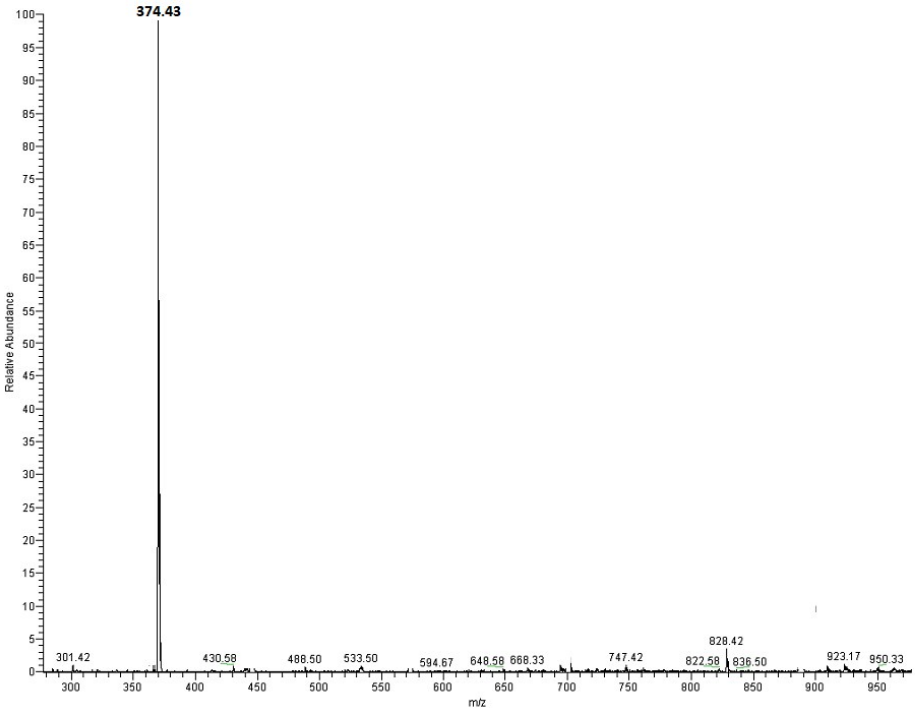

Figure S2. MALDI-TOF spectrum of **EX-1**

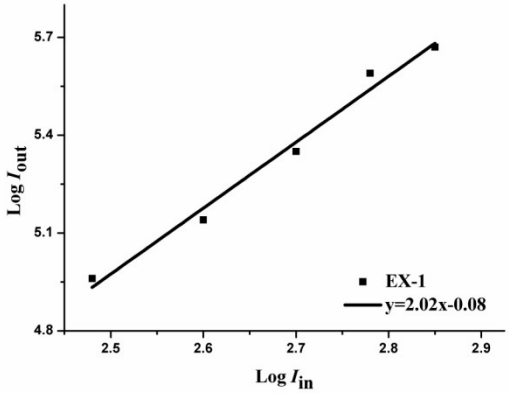

Figure S3. Log–Log linear of the squared dependence of induced fluorescence signal and incident irradiance intensity of **EX-1**

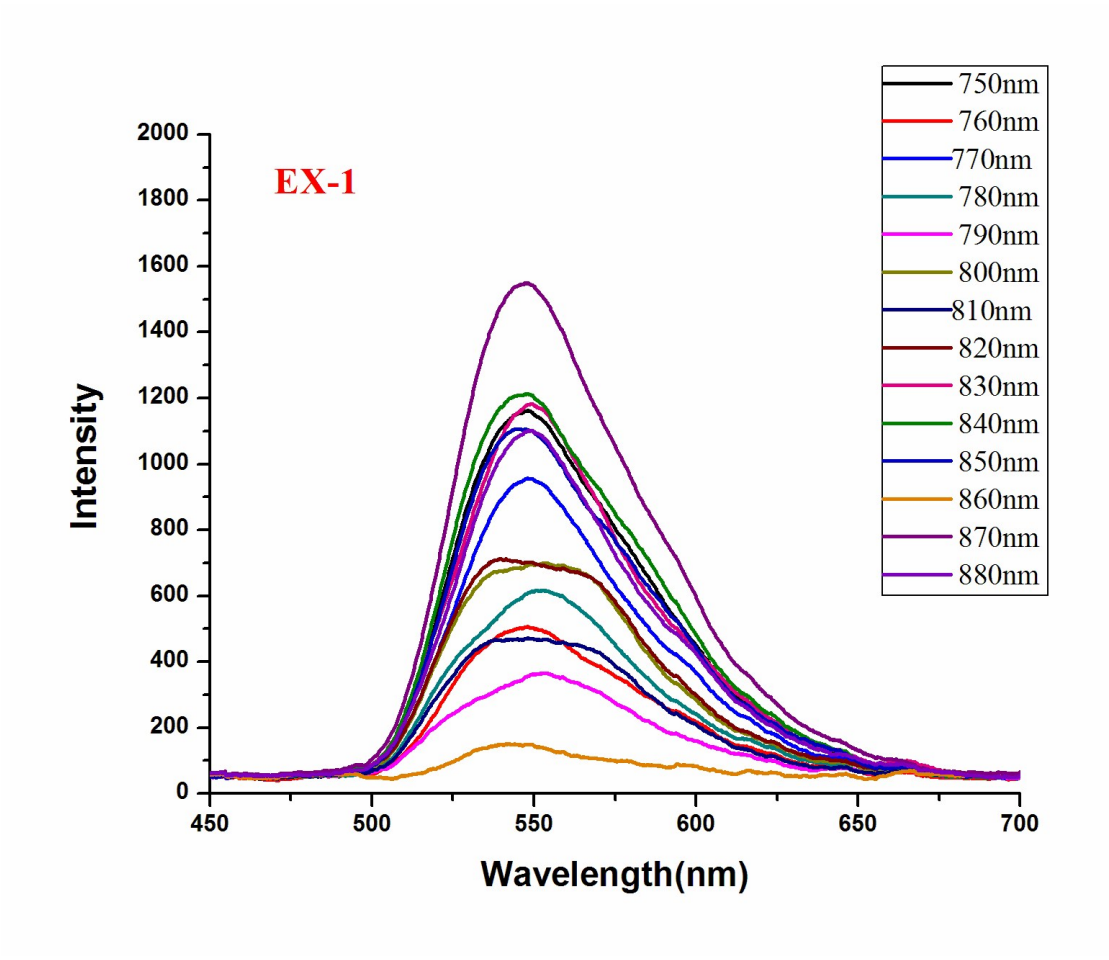

Figure S4. The 2PEF spectra of **EX-1** in DMSO/H_2O pumped by femtosecond laser pulses at 300 mw under different excitation wavelengths.

Table S1. The absorption and fluorescence properties of **EX-1** in different solvents

Compound	Solvents	$\lambda_{max}^{(1a)}$	$\lambda_{max}^{(1f)}$	Φ	τ[ns]
	DCM	424	518	0.1789	0.62
	THF	418	508	0.1257	0.54
EX-1	EtOH	420	533	0.2080	0.95
	CH_3CN	418	527	0.2133	0.96
	DMSO/H_2O	426	528	0.2325	1.02

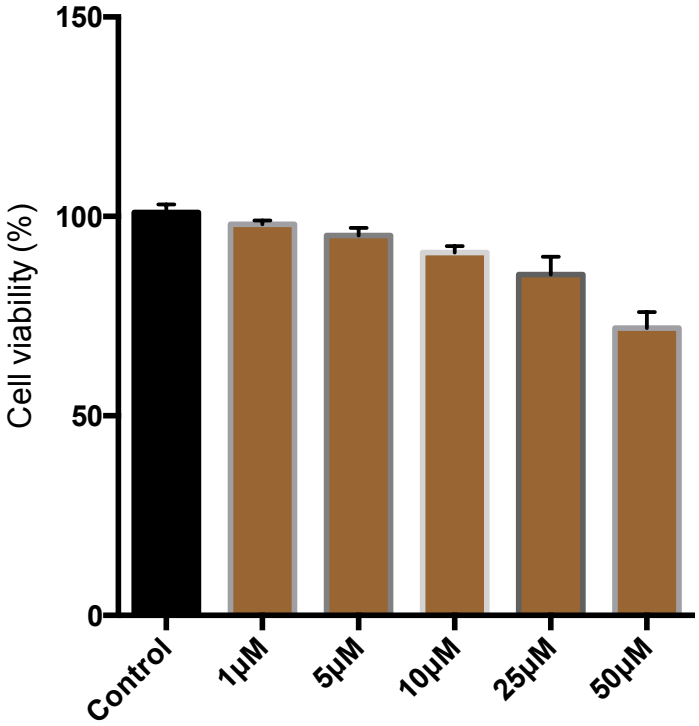

Figure S5. The MTT assay using **EX-1** in different concentration and incubated with HepG2 cells for 24 hours.

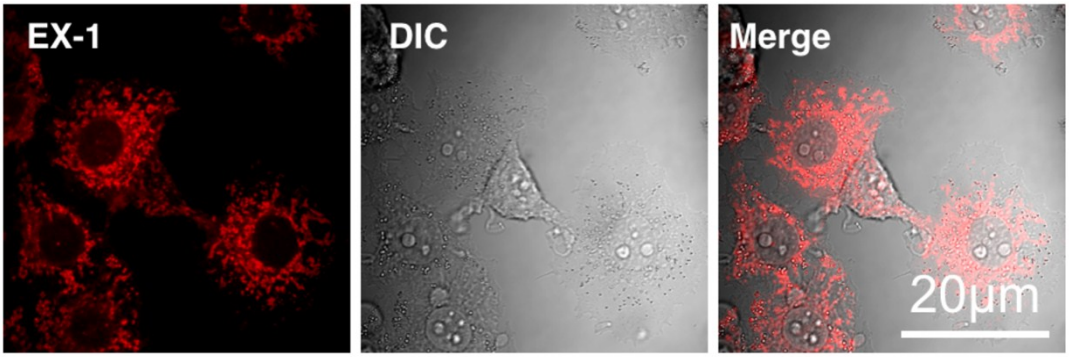

Figure S6. Initial one-photon micrograph using **EX-1** (10 μM) incubated with HepG2 cells for 30 min and imaged under confocal microscopy without fixation.