A Large Stokes' Shift Styryl Pyridinium Derivative with a Stable Green-emission for Mitochondria Imaging in Live Cells

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Supplementary Information

Materials and Method

All chemicals for spectroscopic analyses, cell culture, and bioimaging experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA), and ThermoFisher Scientific (Agawam, MA, USA). Other specialized cell culture and fluorescent confocal microscopy reagents and consumables were purchased from ThermoFisher (Agawam, MA, USA), and Fisher Scientific (Pittsburgh, PA, USA). All spectroscopic analysis experiments were conducted in spectroscopic grade solvents (ThermoFisher). UV–visible spectroscopy studies were performed in an a DS5 (Edinburgh Instruments, Livingston, UK) UV–visible spectrometer at 25 °C. Fluorescence spectroscopy data were acquired using a FS5 photoluminescence spectrometer (Edinburgh Instruments, Livingston, UK) and a HORIBA Fluoromax-4 spectrofluorometer (Horiba Instruments, Irvine, CA) at 25 °C. Fluorescence confocal microscopy imaging studies were conducted using a Leica Stellaris 5 white light laser (WLL) (Mannheim, Germany) confocal microscope. All fluorescence microscopy images were analyzed using the Fiji ImageJ (1.54f) and LasX (Leica Microsystems) image processing applications. All spectroscopy data were processed through the Origin® 2019 software.

Synthesis. To an ethanolic solution (4 mL) of 1-naphthalehyde (312 mg: 2 mmol), ethyl pyridinium iodide **2** (500 mg: 2 mmol) was added and the resulting solution was heated to 65℃ with vigorous stirring followed by the addition of Pyridine (0.25 mL). The resulting bright yellow solution was heated for 12 hours with vigorous stirring. Then the reaction was cooled down to room temperature and added to a flask containing 100 mL of ethyl acetate. The resulting yellow solid was filtered by vacuum and washed further with ethyl acetate (100 mL). EPN was collected as a yellow color solid with 77 % yield. **EPN** was characterized by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry (HRMS). ^{1}H NMR (500 MHz, DMSO) δ 9.05 (d, *J* = 6.8 Hz, 2H), 8.85 (d, *J* = 16.0 Hz, 1H), 8.61 (d, *J* = 8.3 Hz, 1H), 8.50 (d, *J* = 6.8 Hz, 2H), 8.13 – 8.03 (m, 3H), 7.71 – 7.64 (m, 4H), 4.59 (t, *J* = 7.4 Hz, 2H), 1.58 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 153.17, 144.57, 137.56, 133.93, 132.63, 131.55, 131.17, 129.22, 128.84, 127.48, 126.93, 126.36, 126.28, 125.44, 124.88, 124.25, 55.83, 40.54, 40.38, 40.30, 40.21, 40.04, 39.87, 39.71, 39.54, 16.74.HPMS for [M+] found at 260.1406, 261.1620, and 262.1558 (10 ppm error).

Spectroscopy Analysis. Stock solutions of the **EPN** were prepared in molecular biology grade DMSO in either 10 mM For all steady-state measurements (absorbance and emission), the working concentration of the probe was 1×10^{-5} M unless otherwise specified. While acquiring the emission spectra in solution **EPN** was excited at 390 nm and the emissions were collected from 410 nm to 700 nm (unless otherwise specified) at 25 °C. The relative fluorescence quantum yields (ϕ_{fl}) were calculated using Coumarin 153 as the standard (in ethanol) at 400 nm, while considering that the fluorescence quantum yield of Coumarin 153 as 0.554 in ethanol. The following equation was used for fluorescence quantum yield determination [1,2].

$$(\phi_{fl})_{sample} = \phi_{ref} \times (A_{ref}/A_{sample}) \times [(I_{sample})/(I_{ref})] \times (\eta_{sample})^2/(\eta_{ref})^2$$

where A is the absorbance of the sample, I is the integrated fluorescence intensity, and η is the refractive index of the solvent.

Cell Culture and Imaging. HepG2 (Human hepatocellular carcinoma) and A-172 (Human glioblastoma) cells were grown in Dulbecco's modified Eagle's medium (DMEM-high glucose) (Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Penstrep) at 37 °C in a 5% CO_2 humidity-controlled incubator. Cells were seeded in 40 mm Nunc™ glass bottom (#1.5) dishes with a 10 mm bottom diameter. Cells were seeded at a density of 60,000–70,000 cells per well and incubated for 24 h prior to staining. The **EPN** stock solution was made in DMSO at 10 mM and the staining concentration of the **EPN** was 4 μM. For co-staining experiments MitoTracker™ Red FM (1 mM in DMSO) was used. For staining experiments, stained cells were incubated for 30–45 min at 37 °C in a 5% CO_2 humidity-controlled incubator and washed twice with 1 x PBS prior to imaging. The final DMSO percentage during the staining was maintained below 0.20% (*v/v*). All imaging experiments were performed in a Leica Stellaris 5 white light laser (WLL) confocal microscope equipped with HyD S detectors. Co-staining experiments were performed under the analogue (intensity mode) setting and frame acquisition settings, with the following acquisition parameters: **EPN** (λ_{ex} = 405 nm; λ_{em} = 480–560 nm) and MitoTracker™ Red FM (λ_{ex} = 570 nm; λ_{em} = 580-700 nm. The LasX dye assistant platform was used to mitigate the cross-talking/noise during the co-staining experiments. Fluorescence microscopy images were analyzed using the ImageJ (Fiji) software for quantification purposes.

Photostability Evaluation. HepG2 cells stained with **EPN** (4 μM) were excited by a 405 nm diode laser (laser power: 0.4 mW, detector gain: 25%, magnification :40x) at 2-minute intervals while collecting image frames for up to 30 minutes. The average relative fluorescence intensities (recovered) were calculated by analyzing fluorescence microscopy images with ImageJ (Fiji) software. The average fluorescence intensity was plotted as a function of irradiation time to evaluate probe' photostability.

Phototoxicity Evaluation. A-172 ells were stained with **EPN** (4 μM) for 30 minutes. Stained A-172 cells were continuously irradiated with a 405 nm diode laser (laser power: 0.5 mW, detector gain: 25%, magnification :63x oil) at 25-second intervals while collecting bright-field and illumination image frames for a period of 10-minutes. Cellular morphology was analyzed on both the bright-field and illumination image frames to predict any possible phototoxicity effects.

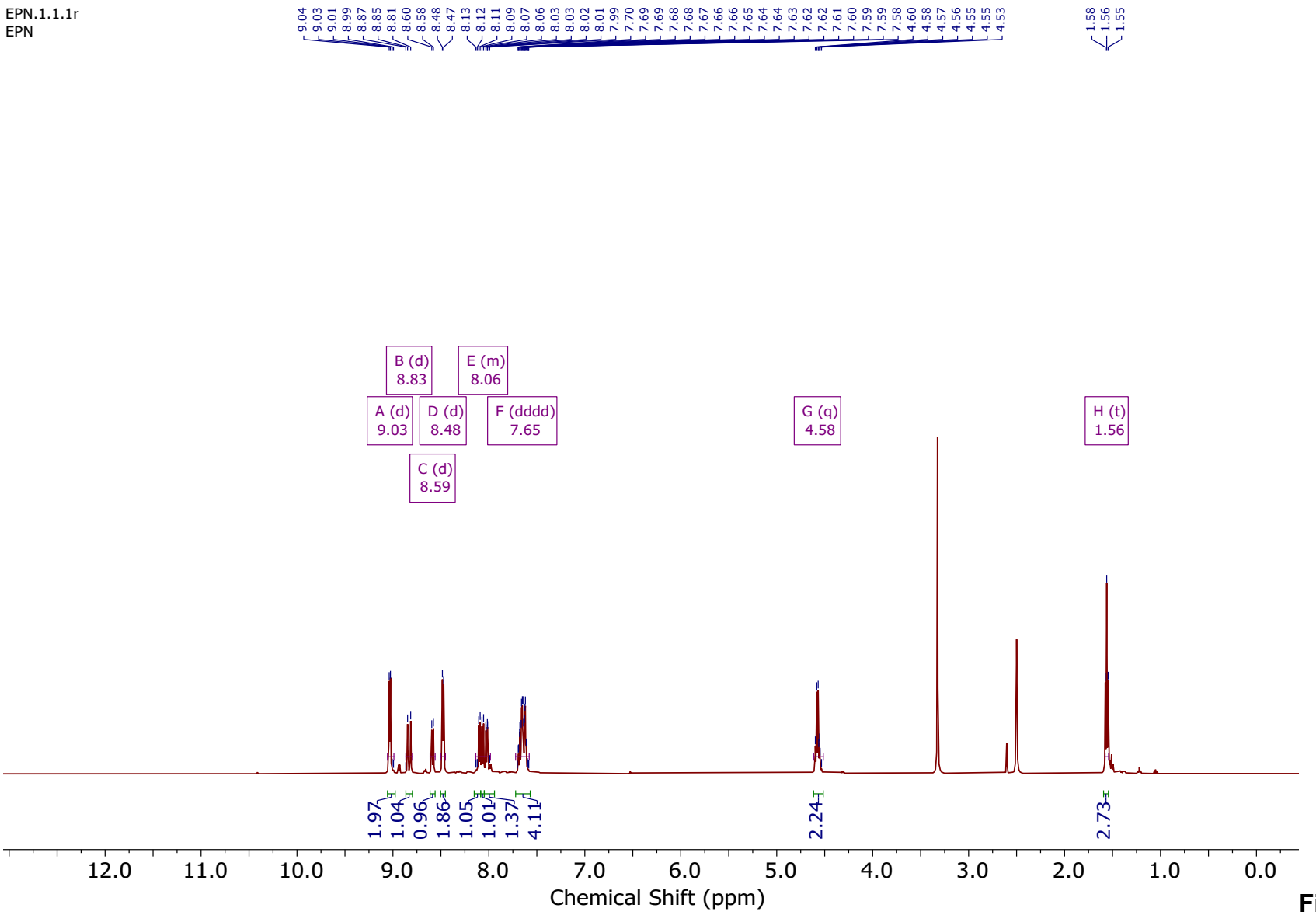
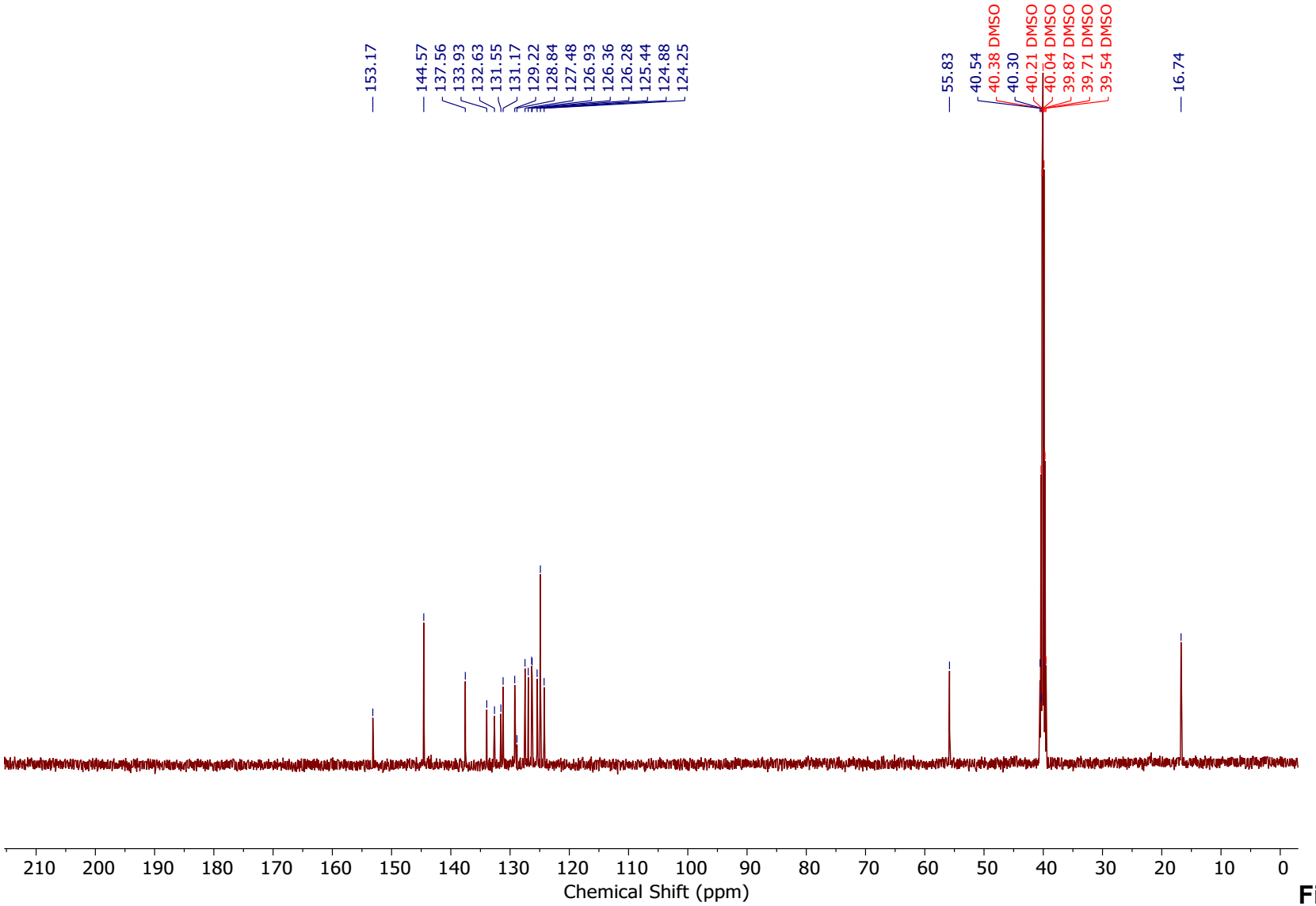


Figure S1.1. ^{1}H NMR spectrum of **EPN** (500 MHz in DMSO-*d6*).



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e S1.2. ^{13}C NMR spectrum of **EPN** (126 MHz in DMSO-*d6*).

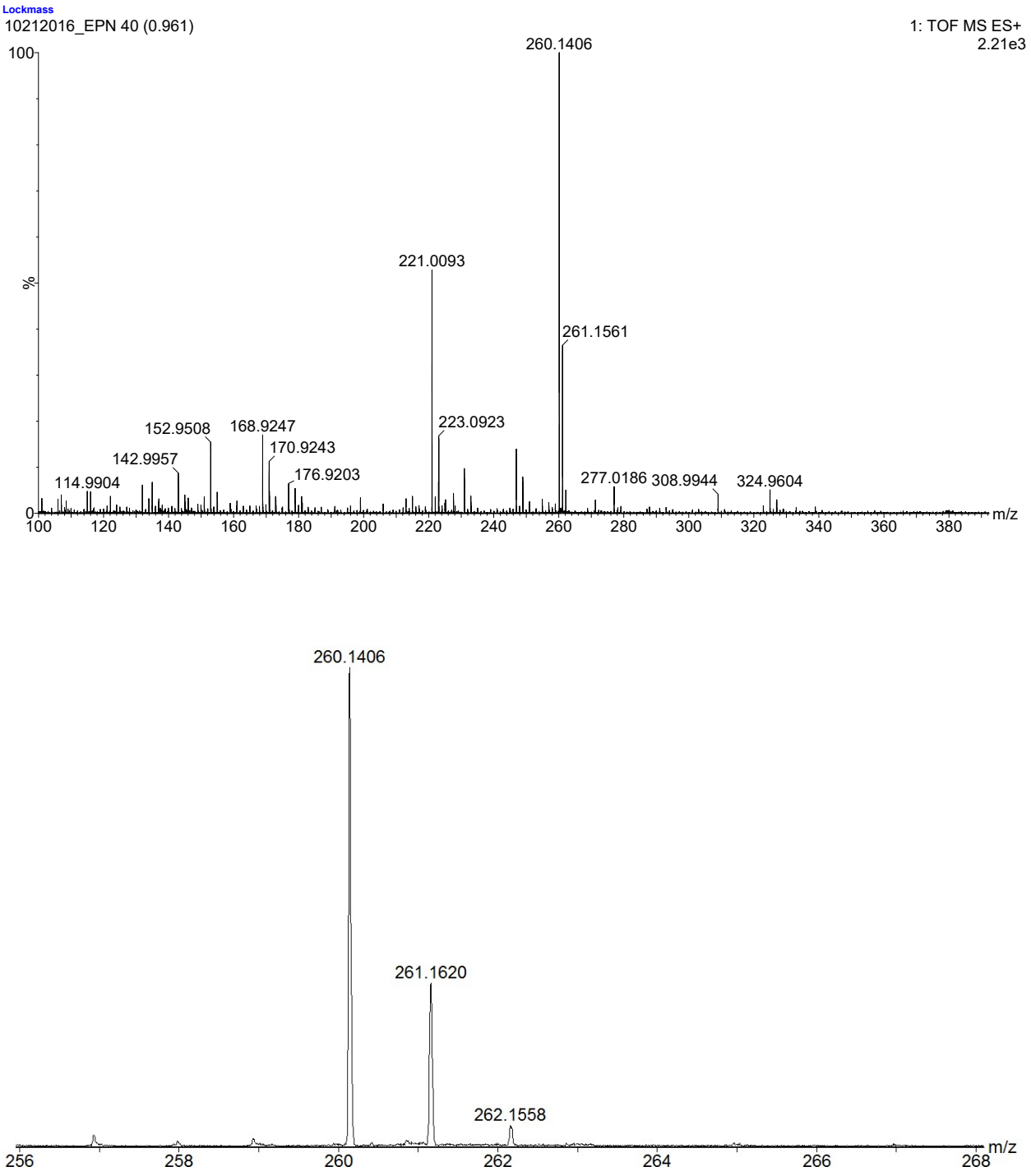

Figure S1.3. HRMS spectra acquired for **EPN** {[$C_{19}H_{18}N^{+}$]}.

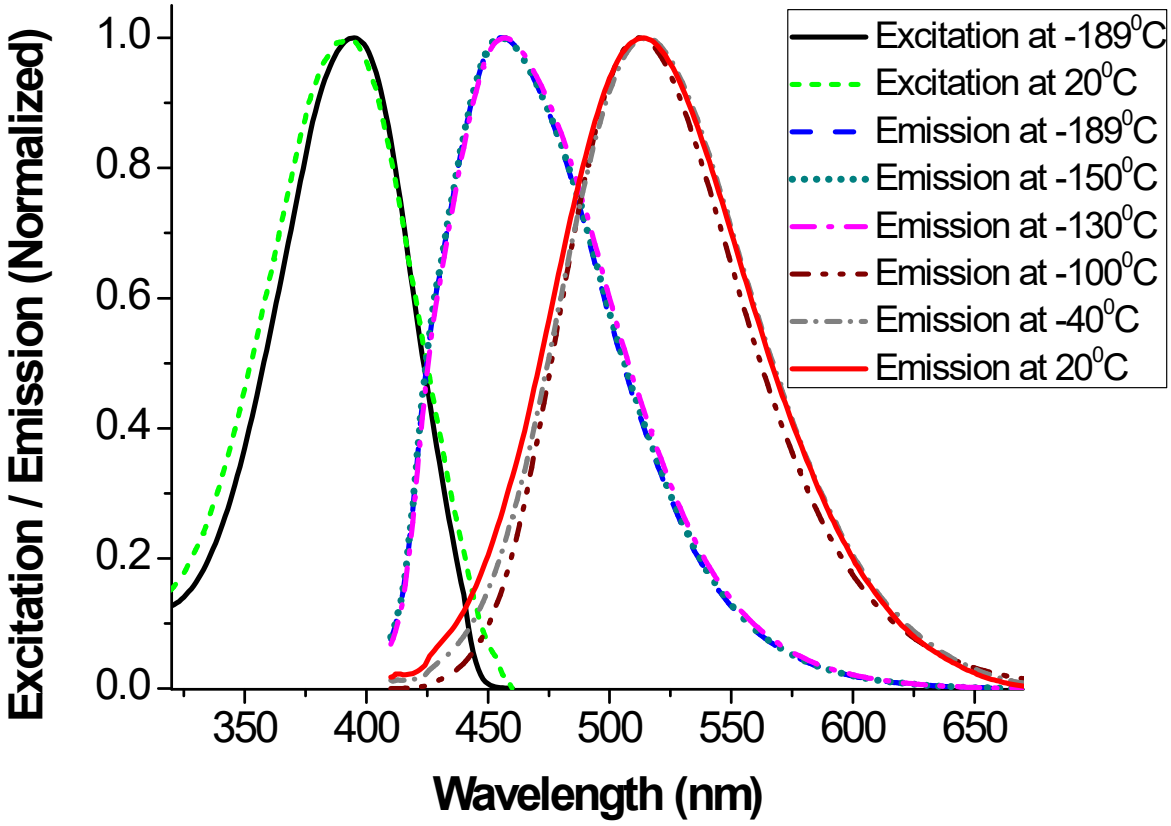

Figure S2. Normalized excitation and fluorescence emission spectra obtained for **EPN** (1×10^{-6} M in Ethanol) at different temperature conditions**.**

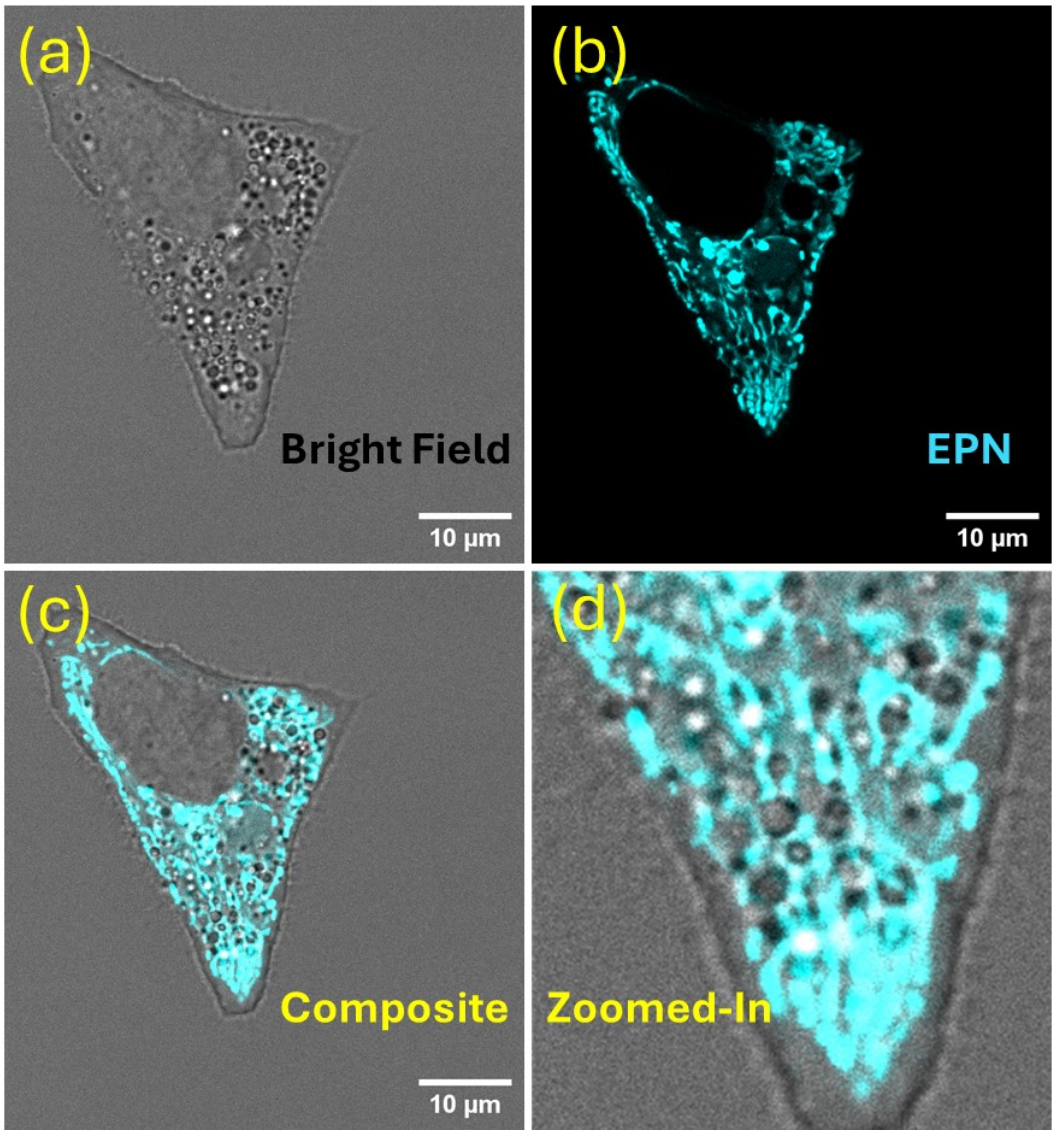

Figure S3. Fluorescence confocal microscopy images of HepG2 cells stained with **EPN** (4 µM) for 30 minutes. Stained cells were excited with 405 nm laser line and the emission was collected from 480 nm to 560 nm.

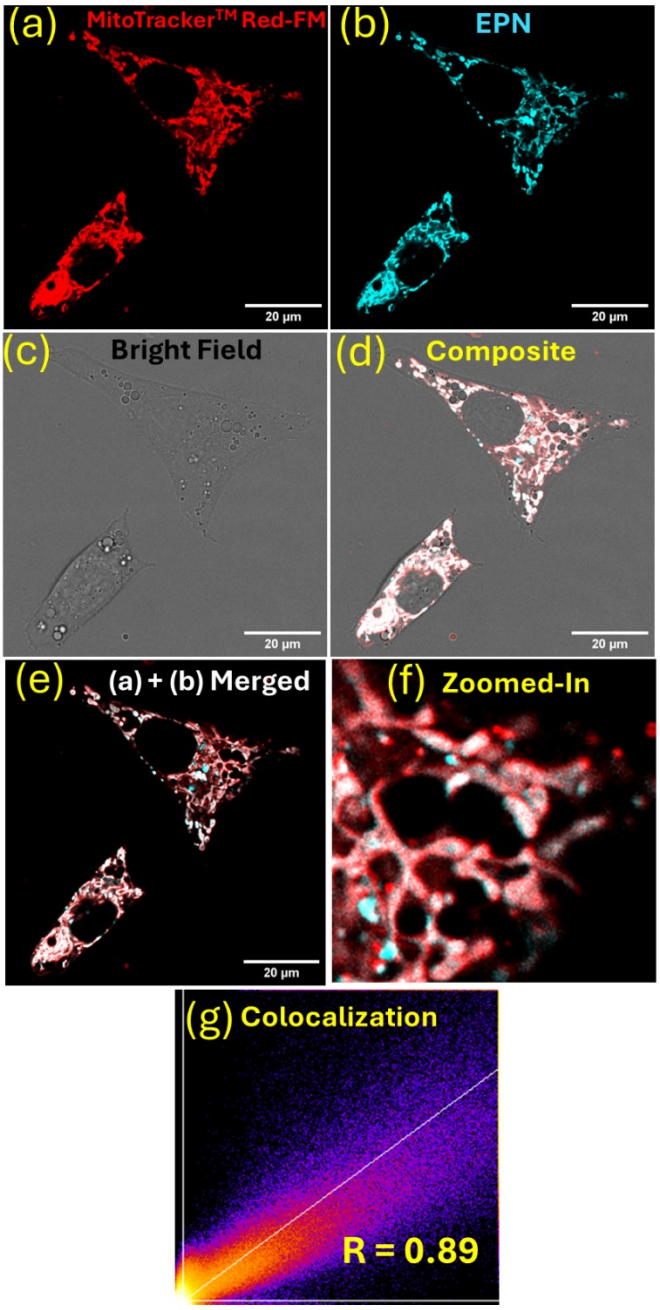

Figure S4. Fluorescence confocal microscopy images of HepG2 cells stained with MitoTracker™ Red FM (200 nM) and **EPN** (4 µM). Images from (a) to (g) represents MitoTracker™ Red FM (a), EPN (b), bright field (c), composite image (d), merged of two dyes (e), a digital zoomed in of e and the colocalization map (g). respectively. MitoTracker™ Red FM was excited at 570 nm and the emissions were collected from 580 nm to 700 nm. **EPN** was excited with 405 nm laser line and the emission was collected from 480 nm to 560 nm.

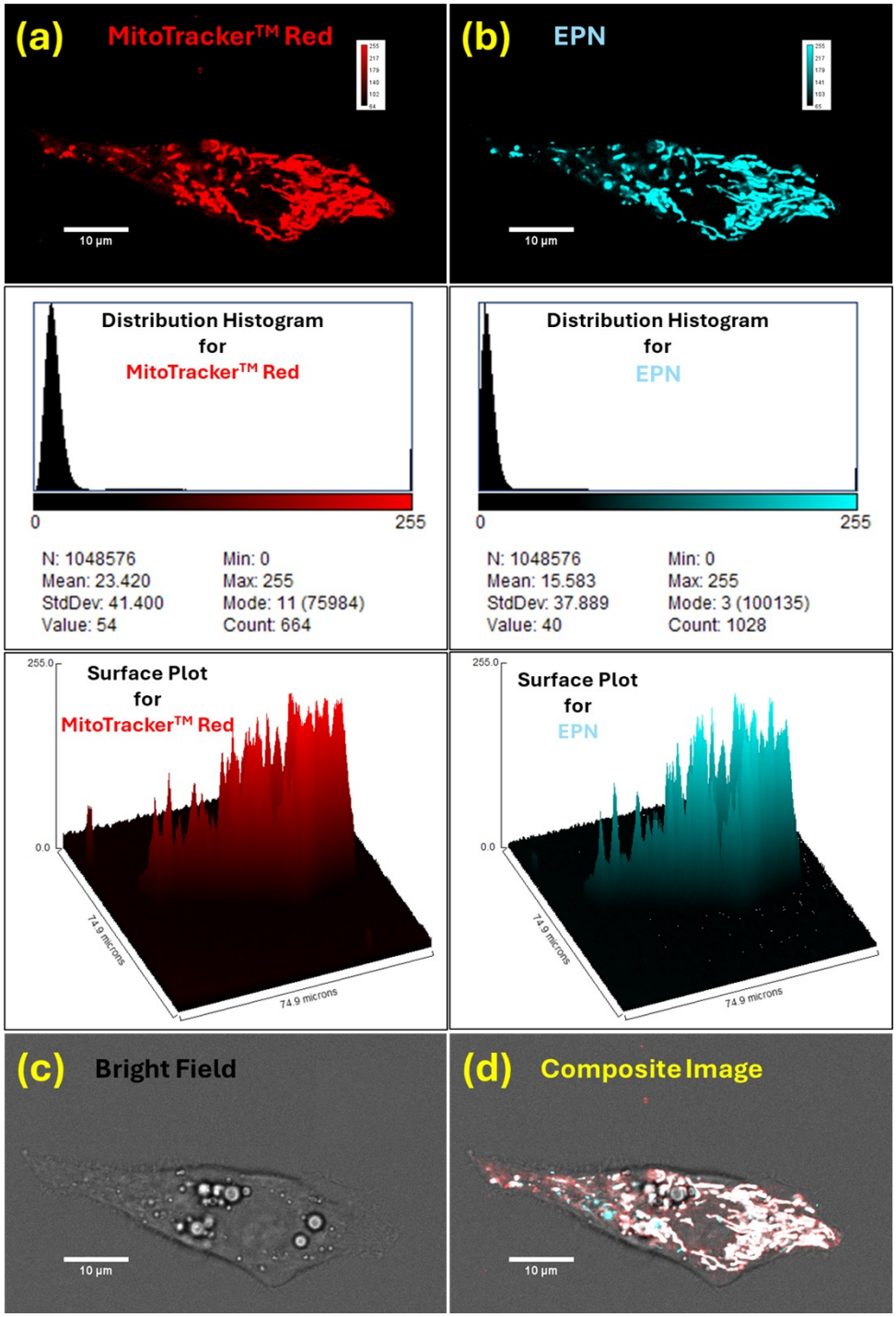

Figure S5. A comparison of florescence intensitieis produced by MitoTracker™ Red FM (a) and **EPN** (b) in HepG2 cells. The corresponding fluorescence intensity histograms and surface plots has been shown for each dye respectively. Figures (c) and (d) bright field (c) and composite image (d) respectively. HepG2 cells were stained with MitoTracker™ Red FM (200 nM) and **EPN** (4 µM) for 30 minutes. MitoTracker™ Red FM was excited at 570 nm and the emissions were collected from 580 nm to 700 nm and **EPN** was excited with 405 nm laser line and the emission was collected from 480 nm to 560 nm.

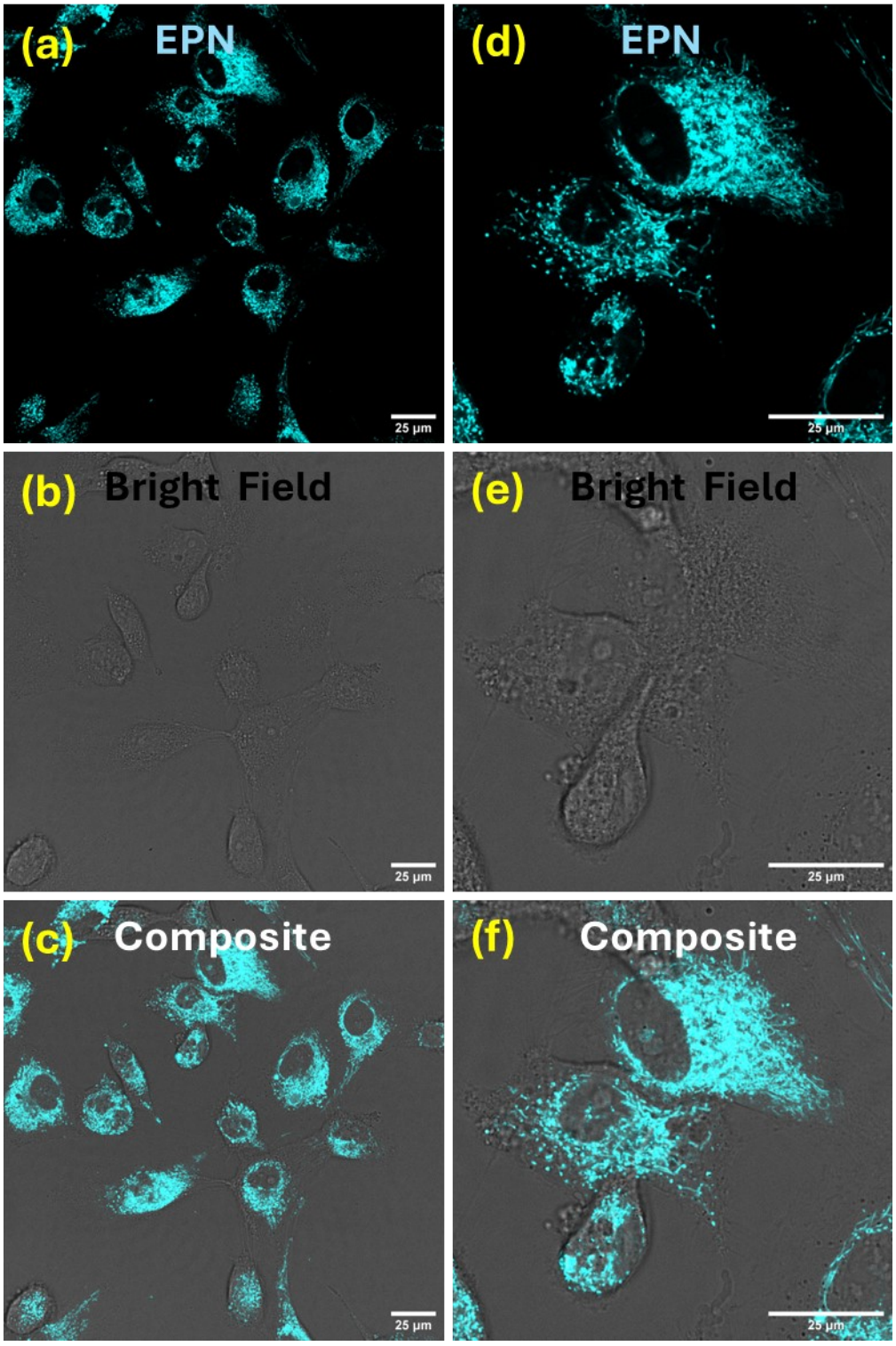

Figure S6. Fluorescence confocal microscopy images of A-172 cells stained with **EPN** (2 µM) for 30 minutes. Stained cells were excited with 405 nm laser line and the emission was collected from 480 nm to 560 nm. Images (a)-(c) are obtained at 63x oil magnification and (d)-(f) represents represents zoomed-in images with 1.8X magnifcation.

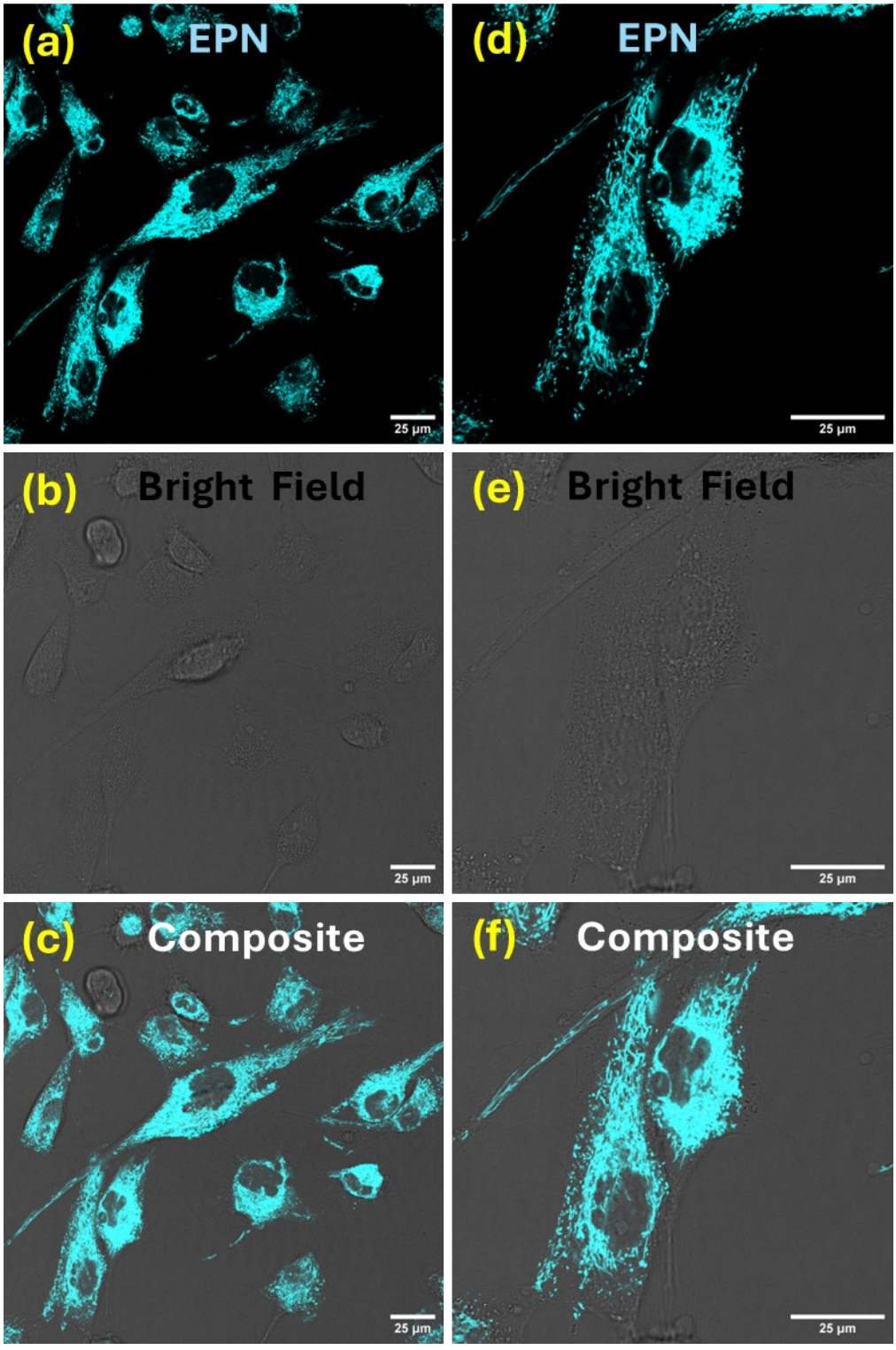

Figure S7. Fluorescence confocal microscopy images of A-172 cells stained with **EPN** (4 µM) for 30 minutes. Stained cells were excited with 405 nm laser line and the emission was collected from 480 nm to 560 nm. Images (a)-(c) are obtained at 63x oil magnification and (d)-(f) represents represents zoomed-in images with 1.8X magnifcation.

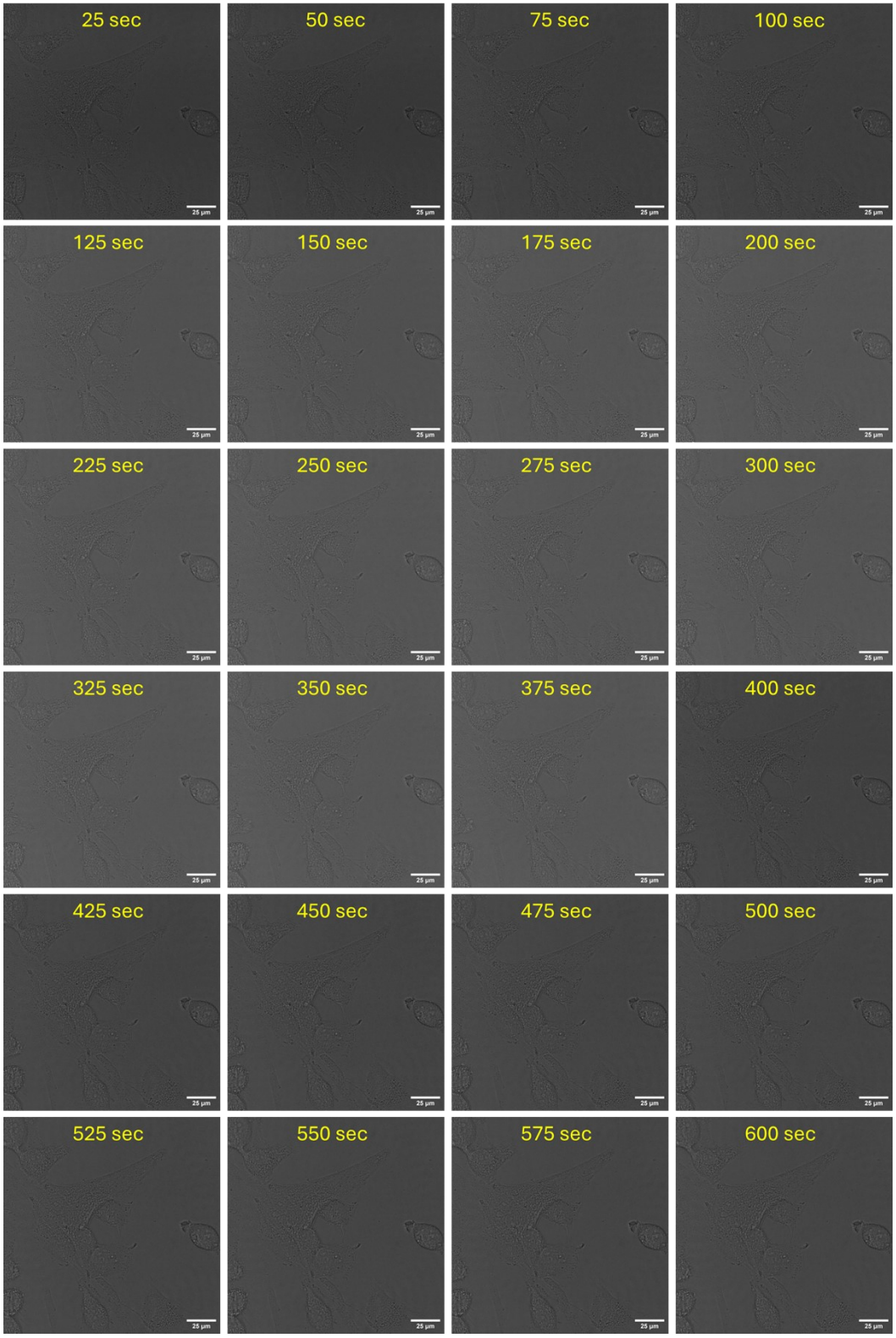

Figure S7.1 Bright-field confocal microscopy images of A-172 cells stained with **EPN** (4 μM) for 30 minutes. Stained cells were excited with 405 nm laser while continuously obtaining images at 25-second time intervals up to 10 minutes.

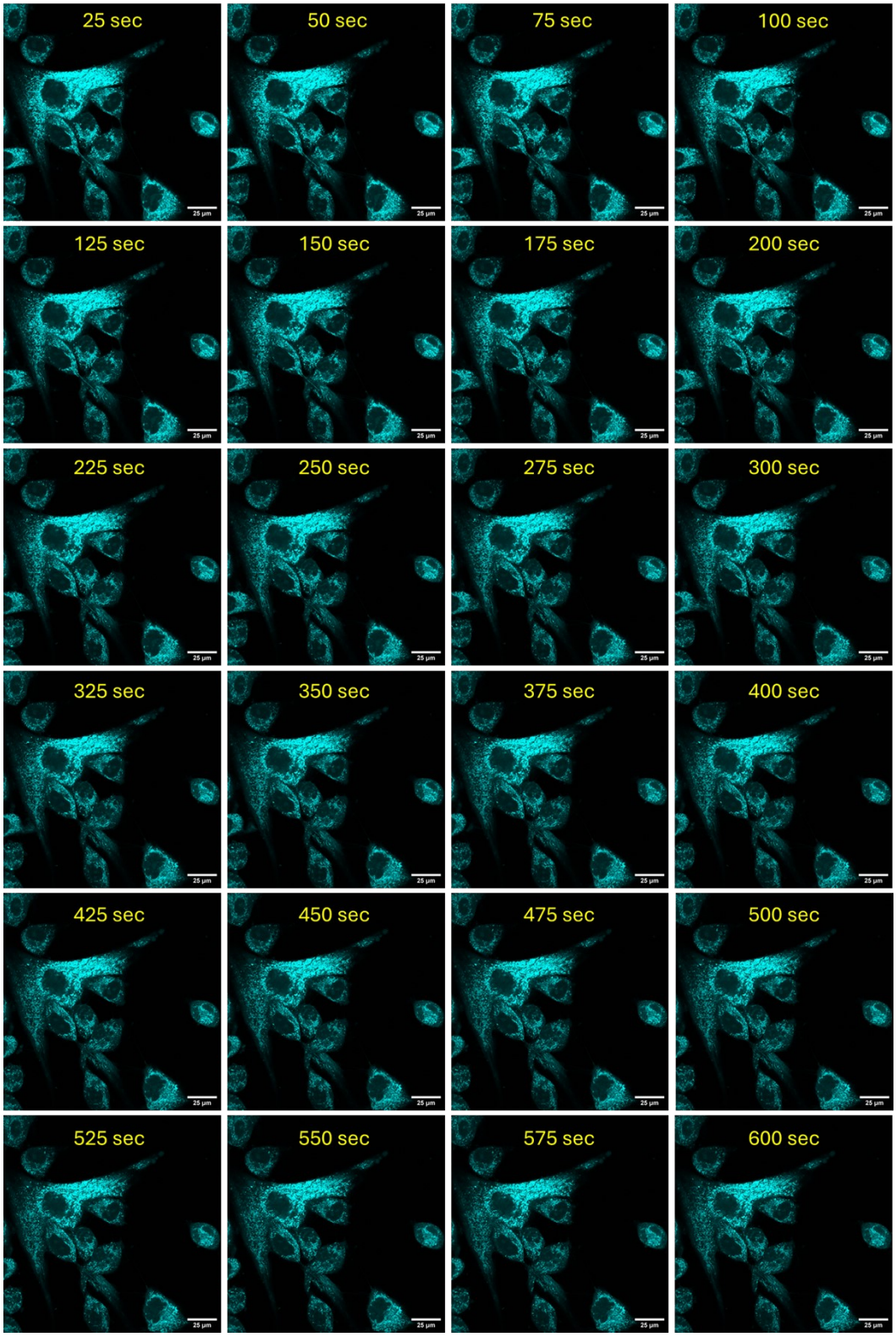

Figure S7.2 Fluorescence confocal microscopy images of A-172 cells stained with **EPN** (4 μM) for 30 minutes. Stained cells were excited with 405 nm laser while continuously obtaining images at 25-second time intervals up to 10 minutes.