Ultrafast Labeling and High-Fidelity Imaging of Mitochondria in Cancer Cells with an Aggregation-Enhanced Emission Fluorescent Probe

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Materials and general instruments

All chemical reagents were obtained from J&K Scientific and were used without further purification. All the solvents for optical spectroscopic studies were HPLC or spectroscopic grade. TLC analyses were performed on silica gel GF 254. Column chromatography purification was carried out on silica gel (200–300 mesh). NMR spectra were recorded using a Bruker AMX-600. Chemical shifts were given in ppm relative to the internal reference TMS, CDCl$_3$ or DMSO-$d_6$ as the internal standard. The following abbreviations were used in $^1$H NMR: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet. High resolution mass spectra were recorded on a Bruker Daltonics Bio TOF mass spectrometer. Fluorescence spectra were obtained using a Horiba Duetta spectrofluorimeter with a 10 mm quartz cuvette. UV-Vis absorption spectra were recorded on a Hitachi PharmaSpec UV-1900 UV-Visible spectrophotometer. The average particle size of the samples was recorded on a Brookhaven Zeta Plus potential analyzer at 25 °C.

MTT assay for the cell cytotoxicity and phototoxicity

The cytotoxicity on HeLa cells was determined by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. HeLa cells were seeded at a density of $7 \times 10^3$ cells per well in 96-well black plates with 100 μL of culture medium and cultured overnight to reach 70–80% confluence. After that the medium was
replaced with 100 μL of fresh medium containing different concentrations of probes (2.5, 5, 10, 15 or 20 μM) and DMSO was used as a vehicle control. Cells were then irradiated by white LED light with a power density of around 5 mW/cm² for 60 min, 120 min or in the dark environment. After 24 h of incubation, 10 μL of 12 mM MTT stock solution (Sigma-Aldrich) mixed with 90 μL of PBS was added to each well for an additional 4 h of incubation. The absorbance was measured at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices). Cell viability (%) was calculated as: \((\text{OD}_{570 \text{ nm sample}}/\text{OD}_{570 \text{ nm control}}) \times 100\%\).

**Cell culture**

HeLa, MDA-MB-231, HepG2, T47D, HEK-293T, HUVEC, and HFF cell lines were kindly provided by the State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University. HeLa cells, MDA-MB-231 cells, HFF cells and HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Life Technologies) and 1% antibiotic-antimyotic (Sigma-Aldrich). HepG2 and T47D cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics-antimyotic. HUVEC cells were cultured in vascular cell basal medium (ATCC) supplemented
with endothelial cell growth kit-BBE (ATCC). All of the cell lines were maintained in the presence of 5% CO₂ in a humidified incubator at 37 °C.

**Imaging**

For confocal imaging, 4 × 10³ cells were seeded in 35-mm glass-bottom dishes and cultured for 24 h. Cells were then incubated with 10 μM of LIQ probes or 5 μM TPE-Ph-In, respectively, for 30 min. After washing with PBS three times, cells were transferred to DMEM medium without phenol red (Life Technologies). Confocal imaging was performed using a Zeiss LSM 880 laser scanning confocal microscope equipped with a Plan-Apochromat 63×/1.4 NA oil objective lens, a photo-multiplier tube and a Gallium arsenide phosphide detector driven by the ZEN software (Carl Zeiss). The 488 nm laser and 560–650 nm emission filter were used for TPE-Ph-In. The 405 nm laser and 420-520 nm (LIQ-1 and LIQ-2) or 500–600 nm (LIQ-3) emission filter were used for probes. The 488 nm laser and 500-550 nm emission filter were used for MTG. Digital images were captured with ZEN software (ZEN 2.5 lite) in grayscale and pseudocolored. Image processing was done using Adobe Photoshop (CS2).

For Co-localization analysis: Image Pro Plus 6.0 software was used for the colocalization analysis by measuring the colocalization of the merged figure in the red and green channels to create calculated correlations
between the images for the Pearson correlation coefficient and to create a color view of scatter plot

To acquire 3D images: reconstruction of Z-stack images on indicated focal planes (190 nm stepwise) were collected with Zeiss LSM 880 laser scanning confocal microscope equipped with a Plan-Apochromat 63×/1.4 NA oil objective lens, a photo-multiplier tube and a Gallium arsenide phosphide detector driven by the ZEN software (Carl Zeiss). For AIQ-3: \(\lambda_{\text{ex}} = 405\) nm, \(\lambda_{\text{em}} = 500-600\) nm. For MTG: \(\lambda_{\text{ex}} = 488\) nm, \(\lambda_{\text{em}} = 500-550\) nm.
Table S1 Optical properties of all probes in solvents with different polarity, in the aggregation state and solid state.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Solvent</th>
<th>( \lambda_{\text{abs}} ) (nm)</th>
<th>( \lambda_{\text{em}} ) (nm)</th>
<th>( \Phi_F ) (%)</th>
<th>( \varepsilon ) (M(^{-1}) \cdot cm(^{-1}))</th>
<th>ΔSS/nm</th>
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<tbody>
<tr>
<td>LIQ-1</td>
<td>DCM</td>
<td>386</td>
<td>477</td>
<td>1.858</td>
<td>10590</td>
<td>91</td>
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<tr>
<td></td>
<td>THF</td>
<td>381</td>
<td>474</td>
<td>1.209</td>
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Absorption and emission maximum were measured in dichloromethane. AEE: aggregation state Fluorescence quantum yields were determined using quinine sulfate in 0.1 M sulphuric acid as a standard (\( \Phi_F = 0.55 \)).
**Fig. S1** (A) UV-vis spectra and (B) fluorescence spectra of **LIQ-1** (10 µM) in DCM, THF, EtOH, DMSO and PBS solution. Excitation wavelength = 386 nm (DCM), 384 nm (DMSO), 380 nm (EtOH), 378 nm (PBS), 381 nm (THF).

**Fig. S2** (A) UV-vis spectra and (B) fluorescence spectra of **LIQ-2** (10 µM) in DCM, THF, EtOH, DMSO and PBS solution. Excitation wavelength = 405 nm (DCM), 402 nm (DMSO), 399 nm (EtOH), 393 nm (PBS), 400 nm (THF).
Fig. S3 (A) UV-vis spectra and (B) fluorescence spectra of LIQ-3 (10 μM) in DCM, THF, EtOH, DMSO and PBS solution. Excitation wavelength = 422 nm (DCM), 418 nm (DMSO), 415 nm (EtOH), 405 nm (PBS), 413 nm (THF).

Fig. S4 (A) Fluorescence spectra of LIQ-3 (10 μM) in DMSO/toluene mixtures. (B) Plots of fluorescence intensity ratio ($I/I_0$) versus toluene fractions of LIQ-3 in mixtures of DMSO/toluene with different toluene fractions. $I_0$, emission intensity in DMSO; $\lambda_{ex} = 418$ nm.
**Fig. S5** (A) Fluorescence spectra of LIQ-1 (10 μM) in DMSO/toluene mixtures. (B) Plots of fluorescence intensity ratio ($I/I_0$) versus toluene fractions of LIQ-1 in mixtures of DMSO/toluene with different toluene fractions. $I_0$, emission intensity in DMSO; $\lambda_{ex} = 384$ nm.

**Fig. S6** (A) Fluorescence spectra of LIQ-2 (10 μM) in DMSO/toluene mixtures. (B) Plots of fluorescence intensity ratio ($I/I_0$) versus toluene fractions of LIQ-2 in mixtures of DMSO/toluene with different toluene fractions. $I_0$, emission intensity in DMSO; $\lambda_{ex} = 402$ nm.
Fig. S7 HeLa cells were incubated with different concentrations of LIQ-1 (2.5, 5, 10, 15, 20 µM) in the absence or presence of white LED light irradiation (5 mW/cm²) for 1 h or 2 h. Cell survival rates was determined by the MTT assay.

Fig. S8 HeLa cells were incubated with different concentrations of LIQ-2 (2.5, 5, 10, 15, 20 µM) in the absence or presence of white LED light irradiation (5 mW/cm²) for 1 h or 2 h. Cell survival rates was determined by the MTT assay.
**Fig. S9** HeLa cells were incubated with different concentrations of LIQ-3 (2.5, 5, 10, 15, 20 µM) in the absence or presence of white LED light irradiation (5 mW/cm²) for 1 h or 2 h. Cell survival rates was determined by the MTT assay.

**Fig. S10** Co-localization of LIQ-1 (10 µM) with TPE-Ph-In (5 µM) in HeLa cells. LIQ-1 (green) and TPE-Ph-In (red) colocalized with each other on mitochondrial structures. The scatter plot displays the correlation between red and green pixel intensities of cells shown in both channels. Degree of colocalization was determined by Pearson’s correlation coefficient ($R_c$). **LIQ-1** ($\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{em}} = 420-520$ nm); **TPE-Ph-In** ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 620-720$ nm).
**Fig. S11** Co-localization of **LIQ-2** (10 μM) with TPE-Ph-In (5 μM) in HeLa cells. LIQ-2 (green) and TPE-Ph-In (red) colocalized with each other on mitochondrial structures. The scatter plot displays the correlation between red and green pixel intensities of cells shown in both channels. Degree of colocalization was determined by Pearson’s correlation coefficient ($R_r$). **LIQ-2** ($\lambda_{ex} = 405$ nm, $\lambda_{em} = 420$-520 nm); TPE-Ph-In ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 620$-720 nm).

**Fig. S12** Fluorescent images of HeLa cells after stained with **LIQ-3** (10 μM, 30 min) and treated without CCCP or treated with 50 μM CCCP for 15 min. $\lambda_{ex} = 405$ nm, $\lambda_{em} = 500$-600 nm.
Fig. 13 Differentiation of cancer cells from normal cells by LIQ-3. (A–G) Fluorescence images of different cancer cells and normal cells stained with LIQ-3 for 30 min. (H) Quantification of relative fluorescence intensity of LIQ-3 in different cell lines relative to that in T47D cells. (Data were from the corresponding images in A-G. A: T47D, B: HeLa, C: MDA-MB-231, D: HepG2, E: HEK-293T, F: HFF, G: HUVEC).
**Fig. S14** Fluorescence images of different cancer cells (T47D, HeLa, MDA-MB-231 and HepG2 cells) with LIQ-3 (10 μM) for 30 min. (λ<sub>ex</sub> = 405 nm, λ<sub>em</sub> = 500-600 nm).
Fig. S15 Fluorescence images of different normal cells (HEK-293T, HFF and HUVEC cells) with **LIQ-3** (10 μM) for 30 min. ($\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{em}} = 500-600$ nm).
Fig. S16 Quantification of relative fluorescence intensity of LIQ-3 in different cell lines relative to that in T47D cells. (A: T47D, B: HeLa, C: MDA-MB-231, D: HepG2, E: HEK-293T, F: HFF, G: HUVEC).
Fig. S17 Dynamic staining images of HeLa cells incubated with LIQ-3 (10 µM) from 1 min to 600 min. Ultrafast labeling of mitochondria could be achieved by LIQ-3 in one minute. ($\lambda_{ex} = 405$ nm, $\lambda_{em} = 500$-600 nm).
Fig. S18 High sensitivity and high-fidelity images of mitochondrial in HeLa cells labeled with different concentration of LIQ-3 from 200 nM to 40 μM for 30 min.
Fig. S19 Fluorescence images of mitochondria in HeLa cells labeled with different concentrations of MTG from 200 nM to 5.0 μM for 30 min. (λ<sub>ex</sub> = 488 nm, λ<sub>em</sub> = 500-550 nm).

**Synthesis and Characterization of fluorescence probes**

To 10 mL of ethyl alcohol, 0.5 mmol dihydroisoquinoline derivative, 0.5 mmol 1,2-diphenylethyne, 0.005 mmol [(Cp*RhCl<sub>2</sub>)<sub>2</sub>, 0.75 mmol silver trifluoroacetate, and 0.5 mmol copper acetate were added. After heated to reflux with magnetic stirring for 30 min under N<sub>2</sub>, the reaction mixture was filtered through diatomite and washed with ethanol. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography on silica gel (DCM to DCM/MeOH = 20/1, v/v) to afford the product.
LIQ-1: light brown solid, 88% yield. $^1$H NMR (600 MHz, CDCl$_3$), δ. 8.68 (d, 1H, $J = 8.4$ Hz), 8.03 (d, 1H, $J = 7.8$ Hz), 7.98 (d, 1H, $J = 7.2$ Hz), 7.93 (t, 1H, $J = 7.2$ Hz), 7.76 (d, 1H, $J = 8.4$ Hz), 7.71 (t, 1H, $J = 7.2$ Hz), 7.62 (t, 1H, $J = 7.2$ Hz), 7.55 (d, 1H, $J = 7.2$ Hz), 7.45-7.44 (m, 2H), 7.37 (m, 3H), 7.33-7.28 (m, 3H), 7.23-7.22 (m, 2H), 4.46 (t, 2H, $J = 6.0$ Hz), 3.30 (t, 2H, $J = 6.0$ Hz). $^{13}$C NMR (100 MHz, DMSO-d$_6$), δ. 157.7 (q, $J = 30$ Hz) (158.0, 157.8, 157.6, 157.4), 153.5, 144.3, 139.1, 138.0, 137.4, 136.2, 135.9, 134.0, 133.7, 133.4, 132.0, 130.9, 130.8, 130.3, 130.2, 129.9, 128.6, 128.4, 128.2, 128.0, 127.5, 126.6, 126.2, 125.3, 124.6, 117.3 (q, $J = 300$ Hz) (120.3, 118.3, 116.3, 114.3), 52.5, 26.5. $^{19}$F NMR (DMSO-d$_6$), δ. -73.51. HRMS (ESI): m/z [M - CF$_3$COO]$^+$ calcd for C$_{29}$H$_{22}$N: 384.1747; found 384.1751.

LIQ-2: Yellow solid, 96% yield. $^1$H NMR (600MHz, CDCl$_3$), δ. 8.63 (d, 1H, $J = 8.4$ Hz), 7.96-7.89 (m, 3H), 7.70 (d, 1H, $J = 8.4$ Hz), 7.37-7.30 (m, 8H), 7.18 (d, 2H, $J = 5.4$ Hz), 7.13 (d, 1H, $J = 8.4$ Hz), 7.10 (s, 1H),
4.40 (s, 2H), 3.98 (s, 3H), 3.27 (s, 2H). $^{13}$C NMR (150MHz, CDCl$_3$), δ. 164.5, 154.3, 144.0, 141.3, 138.7, 135.9, 135.6, 135.5, 133.8, 131.5, 131.0, 130.4, 130.4, 130.3, 130.1, 129.1, 128.5, 128.4, 127.0, 125.1, 119.2, 114.2, 113.2, 56.1, 52.2, 27.8. $^{19}$F NMR (CDCl$_3$), δ.-75.38. HRMS (ESI): m/z [M - CF$_3$COO]$^+$ calcd for C$_{30}$H$_{24}$NO: 414.1852; found 414.1842.

LIQ-3: yellow solid, 93 % yield. $^1$H NMR (600MHz, CDCl$_3$), δ. 8.69 (d, 1H, $J = 8.4$Hz), 7.96 (d, 1H, $J = 7.2$ Hz), 7.91 (1H, t, $J = 7.2$ Hz), 7.72 (d, 1H, $J = 7.8$ Hz), 7.49 (s, 1H), 7.42-7.20 (m, 10H), 7.12 (s, 1H), 4.42 (s, 2H), 4.06 (s, 3H), 3.97 (s, 3H), 3.26 (s, 2H). $^{13}$C NMR (150MHz, CDCl$_3$), δ. 160.6 (q, $J = 30$ Hz) (160.9, 160.7, 160.5, 160.3), 154.5, 154.0, 148.4, 144.2, 138.9, 136.0, 135.5, 133.8, 133.6, 131.5, 130.6, 130.4, 130.3, 130.2, 130.1, 129.1, 128.5, 128.4, 127.2, 125.0, 120.0, 116.1, 110.9, 117.0 (q, $J = 300$ Hz) (120.0, 118.0, 116.1, 114.1), 56.7, 56.6, 52.3, 27.1. $^{19}$F NMR (CDCl$_3$): δ. -74.94. HRMS (ESI): m/z [M-CF$_3$COO]$^+$ calcd for C$_{31}$H$_{26}$NO$_2$: 444.1958; found 444.1947.
$^1$H NMR spectrum of LIQ-1 in CDCl$_3$.

$^{13}$C NMR spectrum of LIQ-1 in DMSO-d$_6$. 
$^{19}$F NMR spectrum of LIQ-1 in DMSO-$d_6$.

HRMS spectrum of LIQ-1.
$^1$H NMR spectrum of LIQ-2 in CDCl$_3$.

$^{13}$C NMR spectrum of LIQ-2 in CDCl$_3$. 
$^{19}$F NMR spectrum of LIQ-2 in CDCl$_3$.

HRMS spectrum of LIQ-2.
$^1$H NMR spectrum of LIQ-3 in CDCl$_3$.

$^{13}$C NMR spectrum of LIQ-3 in CDCl$_3$. 
\(^{19}\)F NMR spectrum of LIQ-3 in CDCl\(_3\).

HRMS spectrum of LIQ-3.

Reference