Supporting information for

A Unique Carbazole-Coumarin Fused Two-Photon Platform: Development of a Robust Two-Photon Fluorescent Probe for Imaging Carbon Monoxide in Living Tissues

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Calculation of fluorescence quantum yield.¹ Fluorescence quantum yield was determined using optically matching solutions of quinine sulfate ($\Phi_f = 0.546$ in 1N $H_2SO_4^2$) as the standard at an excitation wavelength of 350 nm and the quantum yield was calculated using the following equation:

$$\Phi_{\rm s} = \Phi_{\rm r} \left(A_{\rm r} F_{\rm s} / A_{\rm s} F_{\rm r} \right) \left(n_{\rm s}^2 / n_{\rm r}^2 \right)$$

where, s and r denote sample and reference, respectively, A is the absorbance, F is the relative integrated fluorescence intensity, and n is the refractive index of the solvent.

Measurement of two-photon cross section. The two-photon cross section (σ) was determined by using a femtosecond (fs) fluorescence measurement technique. **CC-3-6** were dissolved in DMSO, and **CC-7** and **CC-CO** were dissolved in pH 7.4, 25 mM PBS buffer/DMSO (9:1 v/v), respectively, at a concentration of 5.0×10^{-6} M, and then the two-photon fluorescence was excited at 700-850 nm by using fluorescein in pH = 11 aqueous solution (σ = 32 GM in 810 nm) as the standard, whose two-photon property has been well characterized in the literature.³ The two-photon cross-section was calculated by using $\sigma = \sigma_r (F_t n_t^2 \Phi_r C_r)/(F_r n_r^2 \Phi_t C_s)$, where the subscripts t and r stand for the sample and reference molecules. *F* is the average fluorescence intensity integrated from two-photon emission spectrum, n is the refractive index of the solvent, *C* is the concentration, Φ is the quantum yield, and σ_r is the two-photon cross-section of the reference molecule.



Fig. S1 The absorption spectra of **CC-3** (\blacksquare), **CC-4** (\bullet), **CC-5** (\blacktriangle), and **CC-6** (\bigtriangleup) (5 μ M) in DMSO.

compound	λ_{abs}/nm	λ_{em}/nm	ε _{max} /M ⁻¹ cm ⁻¹	Φ_{f}	σ' (GM)
CC-3	358	436	1.11×10 ⁴	0.65	92.7
CC-4	370		1.69×10 ⁴	0.070	<1
CC-5	378		1.38×10 ⁴	0.023	<1
CC-6	376		1.61×10^4	0.051	<1
CC-7	374	477	0.738×10^{4}	0.51	50.1

Table S1. Photophysical data of CC-3~7.



Fig. S2 The absorption spectra of CC-CO(\blacktriangle) and CC-7 (•) (5.0 µM) in pH 7.4, 25 mM PBS buffer/DMSO (9:1 v/v).

Detection limit: The detection limit was determined from the fluorescence titration data based on a reported method.⁴ According to the result of titration experiment, the fluorescent intensity data at 477 nm were normalized between the minimum intensity and the maximum intensity. A linear regression curve was then fitted to these normalized fluorescent intensity data (Figure S3), and the point at which this line crossed the axis was considered as the detection limit (6.53×10^{-7} M).



Fig. S3 Normalized response of fluorescence signal to changing CORM-2 concentrations.



Fig. S4 a) Pathways for protonolysis of the Pd–C bond as reported previously.⁵ b) Proposed pathways for the conversion of **CC-CO** to **CC-7** in the presence of CO.



Fig. S5 The pH influence on the fluorescence intensity of CC-CO (2.0 μ M) in the absence (**•**) or presence (**•**) of CORM-2.

Kinetic Studies:

The reaction of the probe **CC-CO** (2 μ M) with CORM-2 (50 equiv.) in pH 7.4, 25 mM PBS buffer/DMSO (9:1 v/v) was monitored using the fluorescence intensity at 477 nm. The reaction was carried out at 37 °C. The *pseudo*-first-order rate constant for the reaction was determined by fitting the fluorescence intensities of the samples to the *pseudo* first-order equation (S2):

$$\operatorname{Ln}\left[\left(F_{max} - F_{t}\right) / F_{max}\right] = -k't \qquad (S2)$$

Where F_t and F_{max} are the fluorescence intensities at 477 nm at time *t* and the maximum value obtained after the reaction was complete. *k*' is the *pseudo*-first-order rate constant.



Fig. S6 *Pseudo* first-order kinetic plot of the reaction of **CC-CO** (2.0 μ M) with CORM-2 (50 equiv.) in pH 7.4, 25 mM PBS buffer/DMSO (9:1 v/v). Slope = 0.1107 min⁻¹.



Fig. S7 The fluorescent responses of the probe **CC-CO** (2.0 μ M) to CORM-2 in the presence of various relevant species (100 μ M for H₂O₂, ClO⁻, OH, *t*BuOOH, O₂⁻ and NO.) in pH 7.4, 25 mM PBS buffer/ DMSO (9:1, v/v). 1. CORM-2, 2. CORM-2+ ClO⁻, 3. CORM-2+H₂O₂, 4. CORM-2 +·OH, 5. CORM-2 + *t*BuOOH, 6. CORM-2 + O₂⁻, 7. CORM-2 + NO.



Fig. S8 (A) Photostability profiles of **CC-CO** (2.0 μ M) in the absence [**n**] or presence of UV-irradiated (•) (365 nm), The fluorescence intensities at 477 nm were continuously monitored at time intervals in pH 7.4, 25 mM pH 7.4 PBS buffer / DMSO (9: 1, v/v). Time points represent 0, 5, 10, 15, 20, and 30 min.(B) Chemical stability profiles of **CC-CO** (2.0 μ M) in the absence [**n**] or presence of oxidizing reagents and reducing reagents: blank (**n**), H₂O₂(•), NaClO(**A**), Fe²⁺(**V**), NO(**4**), L-ascorbic acid(**>**). The fluorescence intensities at 477 nm were continuously monitored at time intervals in pH 7.4, 25 mM pH 7.4 PBS buffer / DMSO (9: 1, v/v). Time points represent 0, 10, 20, 30, 40, and 60 min.



Fig. S9 Cytotoxicity assays of **CC-CO** at different concentrations (a: $0 \ \mu$ M; b: $2 \ \mu$ M; c: $5 \ \mu$ M; d: $10 \ \mu$ M; e: $20 \ \mu$ M; f: $30 \ \mu$ M) for HeLa cells.



Fig. S10 Fluorescence imaging of CO in live mammalian cells (A, MCF-7 cells ; B, MNK-28 cells) by **CC-CO** probes: One-photon fluorescent images: (a) Bright-field image of live cells incubated with only **CC-CO** (5.0 μ M) for 30 min; (b) Fluorescence image of (a); (c) Bright-field image of live cells incubated with CORM-2 (200 μ M) for 30 min, then with **CC-CO** (5.0 μ M) for 40 min; (d) Fluorescence image of (c). Excitation at 405 nm. Two-photon fluorescent images: (e) Bright-field image of live cells incubated with only **CC-CO** (5.0 μ M) for 30 min, (f) Fluorescence image of (e); (g) Bright-field image of live cells incubated with CORM-2 (200 μ M) for 30 min, then treated with **CC-CO** (5.0 μ M) for 40 min; (h) Fluorescence image of (g). Excitation at 740 nm. Scale bar = 50 μ m.



Fig. S11 Two-photon fluorescence images of a fresh rat liver slice incubated with 20 μ M **CC-CO** *in the absence of CORM-2* at the depths of approximately 0~180 μ m with a magnification at 20×. Excitation at 740 nm, Scale bar = 150 μ m.



Fig. S12 Two-photon ortho-images of a fresh rat liver slice pretreated with CORM-2 (1 mM) and then incubated with 20 μ M **CC-CO** at the depths of approximately 0~180 μ m with a magnification at 20 ×. Excitation at 740 nm. Scale bar = 150 μ m.

References

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Fig. S13 ¹H NMR spectrum of **CC-2** (CDCl₃).



Fig. S14 ¹³C NMR spectrum of **CC-2** (CDCl₃).



Fig. S15 ¹H NMR spectrum of compound CC-3 (DMSO- d_6).



Fig. S16 13 C NMR spectrum of CC-3 (DMSO- d_6).



Fig. S18¹³C NMR spectrum of CC-3 (CDCl₃).





Fig. S20¹³C NMR spectrum of CC-5 (CDCl₃).



Fig. S22¹³C NMR spectrum of **CC-6** (CDCl₃).



Fig. S24 ¹³C NMR spectrum of CC-7 (CDCl₃).



Fig. S25 ¹H NMR spectrum of CC-7 (DMSO- d_6).



Fig. S26 ¹H NMR spectrum of **CC-CO** (DMSO- d_6).



Figure. S28 ¹H NMR spectra of the isolated product of CC-CO + CO in $CDCl_3$.



Fig. S29 13 C NMR spectra of the isolated product of CC-CO + CO in CDCl₃.