A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

GDCh

www.angewandte.org

## Accepted Article

**Title:** Rational design of a robust fluorescent probe for detecting endogenous carbon monoxide in living zebrafish embryos and mouse tissues

**Authors:** Keyin Liu, Xiuqi Kong, Yanyan Ma, and Weiying Lin



**To be cited as:** *Angew. Chem. Int. Ed.* 10.1002/anie.201707518
*Angew. Chem.* 10.1002/ange.201707518

**Link to VoR:** http://dx.doi.org/10.1002/anie.201707518
http://dx.doi.org/10.1002/ange.201707518

WILEY-VCH

10.1002/anie.201707518


WILEY-VCH

# Rational design of a robust fluorescent probe for detecting endogenous carbon monoxide in living zebrafish embryos and mouse tissues

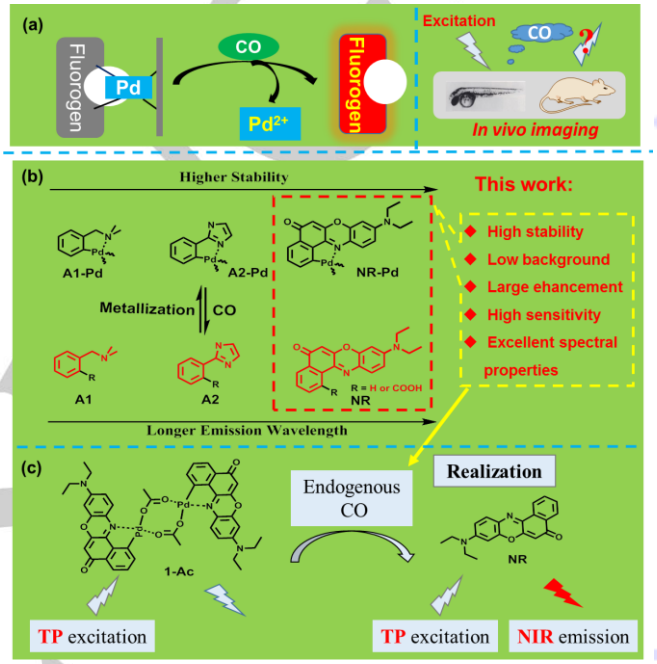
Keyin Liu[§], Xiuqi Kong[§], Yanyan Ma, and Weiying Lin*

Dedication ((optional))

**Abstract:** Carbon monoxide (CO) is one of the most important gaseous signal molecules in biological system. However, investigation of the functions of CO in living animals is restricted by lack of functional molecular tools. To address this critical challenge, herein, we have presented the rational design, synthesis, and *in vivo* imaging studies of a powerful two-photon excited near-infrared fluorescent probe (**1-Ac**) for endogenous CO monitoring. The advantageous features of the new probe include high stability, low background fluorescence, large fluorescence enhancement, high sensitivity, and two-photon excited with emission in near infrared region. Significantly, these merits of the unique probe endow the tracking of endogenous CO in zebrafish embryos and mouse tissues for the first time.

Carbon monoxide (CO) is recognized as one of the most important gas signal molecules for maintaining the physiological activity in biological systems.[1] CO is regarded as a homeostatic molecule with significant cytoprotective, signaling, and inflammation diminishing capabilities in organisms.[2] The main source of endogenously generated CO is from heme oxygenases (HO) mediated heme catabolism.[3] The level of CO in mammalian has significant relation with some severe diseases such as cardiovascular disease,[4] lung disease,[5] septicemia and cancer.[6] Although CO may potentially be used as an indicator in early diagnosis of these diseases,[7] its roles have not been well-defined because of lack of robust functional molecular tools for CO in living animals.

Traditional methods for CO detection includes electronic method,[8] gas chromatography [9] and absorption spectroscopy.[10] Fluorescent detection technique has emerged as a robust method for analysis of CO in biological samples.[11] However, fluorescence detection of endogenous CO in living animals is still impossible due to the short emission wavelengths, large background fluorescence, small fluorescence enhancement, and low sensitivity of the fluorescent probes reported.




**Scheme 1.** (a) General design principle of fluorescent CO probes and the unsolved problem in endogenous CO detection in living animals. (b) The trends of the stability and emission wavelength of the traditional Pd complexes with different core structures (**A1-Pd**, **A2-Pd**) and the new complexes (**NR-Pd**) rationally designed herein. (c) A representative probe (**1-Ac**) of **NR-Pd** with outstanding spectral features such as two-photon (TP) excitation and near infrared (NIR) emission for tracking endogenous CO in living animals and the corresponding sensing mechanism.

Pioneered by Chang *et al*, Pd-contained complexes have been employed as the recognition sites for CO in CO fluorescent probes owing to the fact that the complexes have strong affinity to CO [11a] (**Scheme 1a**). In a Pd-based CO probe, the fluorescence of the dye ligand is typically quenched by the Pd metal atom due to the heavy atom effect. However, CO can interact with Pd at mild conditions to release this metal, which may result in recovery of the fluorescence. As aforementioned, up to date, detection of endogenous CO in living animals remains an unmet challenge. The current fluorescent CO probes such as **A1-Pd** and **A2-Pd** have relatively instable coordinated structures and a comparatively small flexible ligand (**A1-Pd**, **A2-Pd**),[11c, 12] which may lead to high background fluorescence and short emission wavelengths. Thus, we envisioned that it is necessary to design new fluorescent CO probes with advantageous features such as much higher stability and longer emission wavelengths. In general, fluorescent probes with high stability may be favorable for decreasing the background fluorescence, and the probes with longer emission wavelengths

[*] Dr. K. Liu, Dr. X. Kong, Y. Ma, Prof. W. Lin
Institute of Fluorescent Probes for Biological Imaging, School of Chemistry and Chemical Engineering, School of Materials Science and Engineering, University of Jinan, Jinan, Shandong 250022, P.R. China.
E-mail: weiyinglin2013@163.com
[§] These authors contributed equally to this work.
Supporting information for this article is given via a link at the end of the document.((Please delete this text if not appropriate))

are desirable for imaging investigation in living animals.[13] Based on these considerations, in this work, we focused on the Nile Red (**NR**) for design of novel fluorescent CO probes, as it has several merits including a large rigid π structure, potential heteroatoms for complexing with Pd, and a long emission wavelength. Thus, we reasoned that the new type of CO probes, **NR-Pd**, may have much higher stability and longer emission wavelengths when compared to the current CO probes (**Scheme 1b**). These advantageous characters of **NR-Pd** are critical for tracking endogenous CO in living animals. Herein, we engineered the Pd-complex (**1-Ac**) as the representative probe of **NR-Pd** with excellent two-photon excitation and near-infrared emission properties (**Scheme 1c**), which may enable imaging of endogenous CO in living animals.

The preparation of the unique probe **1-Ac** was shown in the Supporting Information. The background fluorescence of **1-Ac** ($\phi$ = 4.69 × $10^{-3}$) at 660 nm in aqueous solution is negligible (**Figure 1a**) due to the strong quenching effect of Pd metal and high stability in aqueous solution. On titrated with the CO donor,CORM-2 [14], significant fluorescence enhancement at 660 nm was observed. The maxima fluorescence turn-on response is about 60 folds ($\lambda_{em}$ = 660 nm) when 2 μM **1-Ac** was incubated in the presence of 100 equiv.CO (**Figures S1 and S2**). Notably, the current Pd-based fluorescent CO probes typically have a turn-on enhancement of roughly only 10 folds (**Table S1**).The large fluorescence enhancement of **1-Ac** can be attributed to the low background fluorescence and high photostability properties in aqueous solution (**Figure S3**).The detection mechanism was verified by HRMS studies (**Figure S4**). The detection limit for CO was determined to be 5.0 × $10^{-8}$ M (**Figure 1b**), which is much lower than that of other Pd-based probes (**Table S1**),suggesting that the new probe is extremely sensitive to trace amount of CO *in vitro*, and may have the potential for detection of endogenous CO generated in living organisms.

The selectivity of **1-Ac** toward CO was investigated (**Figure 1c**), While **1-Ac** showed large fluorescence enhancement at 660 nm only when the CO donor was added, other bioactive small molecules such as reactive oxygen species ($H_2O_2$, $OH^{\cdot}$, $CH_3COOOH$, $ClO^-$), reactive nitrogen species (NO, $NO_2^-$, $ONOO^-$), and reactive sulfur species ($HS^-$, $SO_3^{2-}$, $S_2O_3^{2-}$) exhibited negligible fluorescence response under the same conditions.

The above studies demonstrate that **1-Ac** possessed high selectivity, excellent photostability, especially a large enhancement signal, and low limit of detection. Besides, the probe **1-Ac** exhibited no marked cytotoxicity in HeLa and Raw264.7 cells by the MTT assays (**Figure S5**).

Initially, the monitoring of exogenous CO was performed in HeLa cells using **1-Ac**. The control cells treated with only 2 or 5 μM **1-Ac** showed no fluorescence (**Figures S6a and S6c**). By contrast, the cells loaded with **1-Ac** and CORM-2 displayed remarkable NIR fluorescence in the one-photon mode (**Figures S6b and S6d**), revealing that **1-Ac** has the ability of imaging exogenous CO in the living cells. Notably, the time-dependent results in the cells suggest that the fluorescence is dependent on the concentrations of CO (**Figures S7 and S8**).

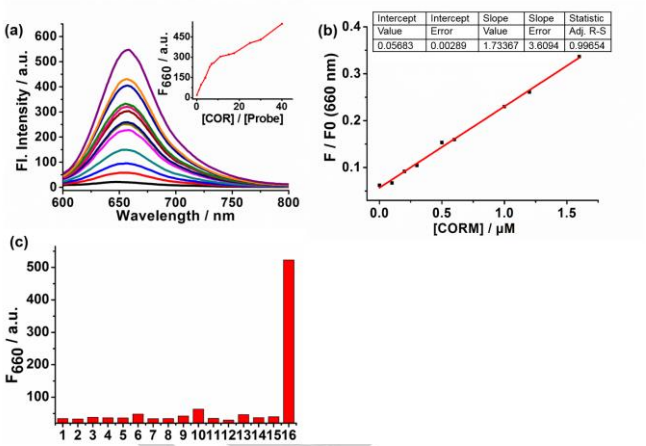



**Figure 1.** The fluorescence response of **1-Ac** with CO. (a) 2 μM of **1-Ac** titrated with increasing amount of CO in PBS (10 mM PBS, pH 7.4, with 5% DMSO) for 30 min. The inset in (a) is the intensity change of **1-Ac** at 660 nm with the ratio of CORM-2 over the probe (b) Linear fit of the fluorescence intensity change at 660 nm with CORM-2 concentration. (c) The selectivity experiment of **1-Ac** with CO. The fluorescence intensity change at 660 nm of 2 μM of **1-Ac** interacted with 100 eq. of different species for 30 min. Numbers 1-16 represent $H_2O_2$, $OH^{\cdot}$, $CH_3COOOH$, $ClO^-$, TBHP, $^tBuOOH$, NO, $NO_2^-$, $ONOO^-$, Cys, HCy, GSH, $HS^-$, $SO_3^{2-}$, $S_2O_3^{2-}$, CO, respectively, $\lambda_{ex}$ = 580 nm.

The two-photon images of **1-Ac** were obtained synchronously (**Figures S6, S7, and S8**). The results are nicely consistent with those obtained in the one-photon mode; no two-photon fluorescence was observed in the control cells incubated with only **1-Ac**. However, the cells loaded with **1-Ac** and CORM-2 exhibited a strong two-photon signal. Taken together, **1-Ac** is capable of detecting exogenous CO in the living cells under both the one- and two-photon modes.

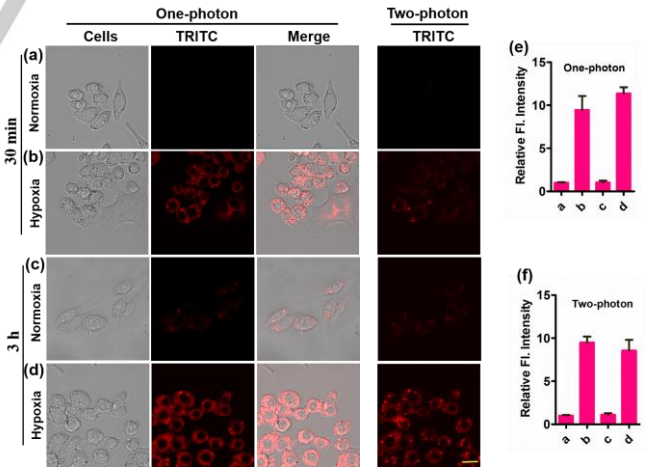



**Figure 2.** Imaging of endogenous CO using **1-Ac** in Raw 264.7 cells by one-photon (left) and two-photon modes (right). (a) and (c) The cells were pre-incubated in a normoxia incubator (37 °C, 5% $CO_2$, 95% air) for 24 h, then treated with 5 μM **1-Ac** for 30 min and 3 h, respectively; (b) and (d) The cells were pre-incubated in a hypoxia incubator (37 °C, 5% $CO_2$, 2% $O_2$ / 98% $N_2$) for 24 h, then treated with 5 μM **1-Ac** for 30 min and 3 h, respectively; (e) Quantified fluorescence relative intensities in the one-photon mode; (f) Quantified fluorescence relative intensities in the two-photon mode. Error bars represent standard deviation (±S.D.). n = 3, the statistical analysis was

performed from three separate biological replicates. One-photon mode: $\lambda_{ex}$ = 561 nm; $\lambda_{em}$ = 570 - 620 nm. Two-photon mode: $\lambda_{ex}$ = 760 nm; $\lambda_{em}$ = 570 - 620 nm. Scale bar: 20 μm.

We next turned our attention to detect endogenous CO in living cells with the new probe. [15] Raw 264.7 cells kept in a normoxia incubator (5% $CO_2$, 95% air) for 24 h showed almost no fluorescence after treatment with 5 μM **1-Ac** for 30 min (**Figure 2a**). However, the cells placed in a hypoxia incubator (98% $N_2$ / 2% $O_2$) exhibited remarkable NIR fluorescence after incubation with **1-Ac** for 30 min in both one- and two-photon modes (**Figure 2b**), suggesting that **1-Ac** is suitable for tracking endogenous CO in living cells under both one- and two-photon modes. Moreover, the time-dependent fluorescence changes were also investigated (**Figure S9**). Only weak fluorescence was obtained in the normoxia cells after 3 h incubation (**Figure 2c**). By contrast, NIR fluorescence in Raw 264.7 cells increased upon extensive incubation time (**Figure 2d**). The quantified intensities with all groups in both one- and two-photon modes (**Figure 2e and 2f**) illustrate that **1-Ac** may be employed as an effective tool for monitoring the endogenous CO.

Based on the above studies, we then attempted to visualize changes of CO in living animals with **1-Ac**. We firstly focused on imaging of exogenous CO in larval zebrafish. The control larval zebrafish gavaged with only 10 μM **1-Ac** showed almost no fluorescence (**Figure S10**). By contrast, larval zebrafish gavaged with **1-Ac** and CORM-2 exhibited distinct NIR fluorescence in the intestine under both the one-photon and two-photon fluorescence, indicating that the new probe can detect CO in living zebrafish (**Figure S10**).

The possibility of **1-Ac** to detect endogenous CO *in vivo* was then investigated. Nearly no fluorescence appeared in the control zebrafish embryos incubated with **1-Ac** under the normoxia conditions (**Figures 3a and 3c**). We then performed a series of experiments with zebrafish embryos under the hypoxia to produce endogenous CO. The zebrafish embryos incubated with **1-Ac** under the hypoxia conditions elicited a significantly higher NIR fluorescence readout under one- and two-photon modes (**Figures 3b and 3d**), suggesting that **1-Ac** has the ability of visualizing endogenous CO in the living system. Notably, the zebrafish embryos treated with 5 μM **1-Ac** exhibited stronger fluorescence than the group treated with 2 μM **1-Ac** (**Figures 3e and 3f**).

We further investigated whether **1-Ac** could image CO in living tissue slices. The mouse liver tissue slices treated by only 10 μM **1-Ac** showed negligible fluorescence at the depth of 90 μm by the two-photon mode (**Figure S11a**), while the tissue slices were pre-incubated with CORM-2 for 30 min and further treated with **1-Ac** exhibited dramatic two-photon fluorescence at a depth of 90 μm (**Figure S11b**), and the penetration of fluorescence reached up to 130 μm (**Figure S11c**). Notably, the two-photon fluorescence spectral profiles were obtained in the living liver tissue slices (**Figures S12** and **S13**). These results indicate that **1-Ac** is capable of imaging CO in the living tissue slices.

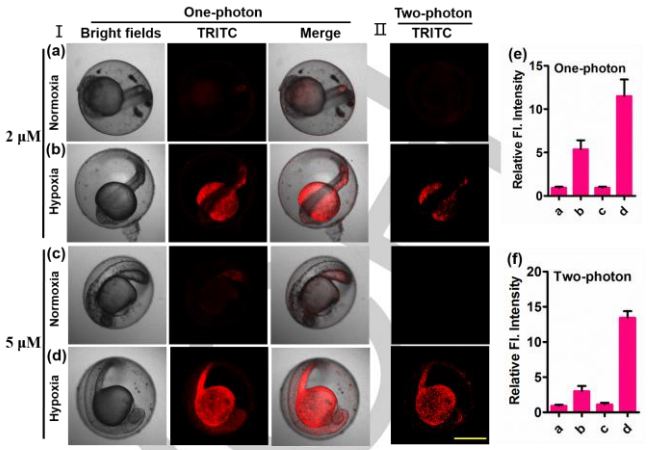



**Figure 3.** Imaging of endogenous CO using **1-Ac** in zebrafish embryos by one-photon (left) and two-photon modes (right). (a) and (c) Zebrafish embryos were pre-incubated in the normoxia incubator for 12 h, then treated with 2 or 5 μM **1-Ac** for 30 min, respectively; (b) and (d) Zebrafish embryos were pre-incubated in the hypoxia incubator for 12 h, then treated with 2 or 5 μM **1-Ac** for 30 min respectively; (e) Quantified fluorescence relative intensities in one-photon mode; (f) Quantified fluorescence relative intensities in two-photon mode. Error bars represent standard deviation (±S.D.). n = 3, the statistical analysis was performed from three separate biological replicates. One-photon mode: $\lambda_{ex}$ = 561 nm; $\lambda_{em}$ = 570 - 620 nm. Two-photon mode: $\lambda_{ex}$ = 760 nm; $\lambda_{em}$ = 570 - 620 nm. Scale bar: 500 μm.

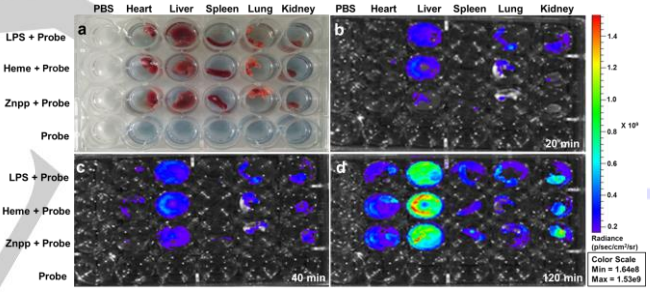



**Figure 4.** NIR fluorescence imaging of endogenous CO with the probe **1-Ac** in mouse organs by an *in vivo* imaging system. (a) Bright field images of mouse organs treated with 10 μM probe (500 μL) in a 24-well plate, **Column 1**: Control PBS; **Column 2** – **Column 6**: the heart, liver, spleen, lung, kidney incubated with 10 μM **1-Ac**, respectively. **Raw 1** – **Raw 3**: organs taken from the mice pretreated by LPS, Hemin, or ZnPP for 4 days, and then further incubated with 10 μM **1-Ac**; **Raw 4**: 10 μM **1-Ac** only. (b – d) Merged images of fluorescence and brightfields of the organs treated with 10 μM probe for 20, 40, and 120 min, respectively. $\lambda_{ex}$ = 580 nm, $\lambda_{em}$ = 660 nm.

Next, we evaluated the ability of **1-Ac** to sense CO in living organs. It is known that LPS or hemin could induce the expression of HO-1, which can facilitate production of CO. However, ZnPP may inhibit the expression of HO-1, resulting in depressing the production of CO.[5a] The mice were stimulated by LPS, hemin, or ZnPP for 4 days, respectively, then the organs were isolated and loaded with **1-Ac** for different times, and finally subjected to imaging. As shown in **Figure 4**, there was almost no fluorescence in the PBS groups treated with **1-Ac**. However, the fluorescence signals in livers, lungs, and kidneys stimulated by LPS or hemin are significantly stronger than those treated with ZnPP, which confirms the generation of CO by LPS,

hemin, and depression of CO by ZnPP, demonstrating that **1-Ac** could detect CO in the organs. Importantly, fluorescence became higher with the extended incubation time, especially in the livers, which may reflect that the distributional difference of CO in the distinct organs and the liver possibly produced more CO than the other organs (**Figure S14**). Taken together, **1-Ac** can effectively track CO in the living organs.

Finally, we assessed the ability of **1-Ac** to image CO in the living mice. The mice were injected with **1-Ac** by an intraperitoneal manner, followed by an injection of CORM-2. The mice displayed essentially no fluorescence treated with only **1-Ac** (**Figure 5a**). By contrast, the fluorescence signals were obtained after injection of CORM-2 within 2 min, demonstrating that **1-Ac** can sense CO in living mice rapidly. The fluorescence signal is time-dependent, and it reached saturation after 30 min with a roughly 7-fold enhancement relative to the control group (**Figure 5b**). Therefore, the studies indicate that **1-Ac** can detect CO in the living whole animals.

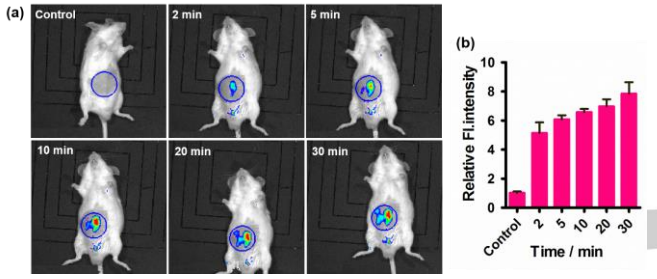



**Figure 5.** *In vivo* fluorescence imaging of CO in the living mice using **1-Ac**. (a) time-dependent fluorescence images of CO in the living mice. Control group: only 100 μL of 10 μM **1-Ac** was injected. Other groups: 100 μL of 10 μM **1-Ac** was injected, then 100 μL of 100 μM CORM-2 was injected and imaged for 2, 5, 10, 20, and 30 min, respectively. (b) The relative average fluorescence intensity change with time in the blue circle area. Error bars represent standard deviation (±S.D.). n = 3, the statistical analysis was performed from three separate biological replicates. $\lambda_{ex}$ = 580 nm, $\lambda_{em}$ = 660 nm.

In summary, we have engineered the first two-photon excited near infrared fluorescent probe suitable for endogenous CO imaging in living animals through rational design. The new probe exhibited highly favorable properties, such as two-photon excitation, near infrared emission, high stability, low background fluorescence, large fluorescence enhancement, and low detection limit. These superior features of the novel probe enable us tracking of CO in both zebrafish embryos and living mice for the first time. The fluorescence imaging studies of CO illustrated herein may provide guide for further exploring the functions of CO in living animals.

## Acknowledgements

This work was financially supported by NSFC (21472067, 21672083, 61605060), NSF of Shandong Province (ZR2015PB016, ZR2017PH051), Taishan Scholar Foundation (TS 201511041) and the startup fund of University of Jinan (309-10004, 160100137).

**Keywords:** Fluorescent probe • endogenous CO detection • *in vivo* imaging • near infrared emission •

[1] a) S. W. Ryter, A. M. K. Choi, *Am. J. Respire. Cell Mol. Boil.* **2009**, *41*, 251-260; b) E. O. Owens, *Clin. Biochem.* **2010**, *43*, 1183-1188.
[2] a) D. G. Levitt, M. D. Levitt, *Clin. Pharmacol.* **2015**, *7*, 37-56; b) L. Wu, R. Wang, *Pharmacol. Rev.* **2005**, *57*, 585-630; c) G. D'Amico, F. Lam, T Hagen, S. Moncada, *J. Cell Sci.* **2006**, *119*, 2291-2298.
[3] I. Bauer, B. H. J. Pannen, *Crit. Care* **2009**, *13*, 220.
[4] A. Nakao, D. J. Kaczorowski, R. Sugimoto, T. R. Billiar, K. R. McCurry, *J. Clin. Biochem. Nutr.* **2008**, *42*, 78-88.
[5] S. W. Ryter, A. M. K. Choi, *Transl. Res.* **2016**, *167*, 7-34.
[6] R. Motterlini, L. E. Otterbein, *Nat. Rev. Drug Discov.* **2010**, *9*, 728-743.
[7] a) D. Basudhar, L. A. Ridnour, R. Cheng, A. H. Kesarwala, J. Heinecke, D. A. Wink, *Coordin. Chem. Rev.* **2016**, *306*, 708-723; b) D. Wang, E. Viennois, K. Ji, K. Damera, A. Draganov, Y. Zheng, C. Dai, D. Merlin, B Wang, *Chem. Commun.* **2014**, *50*, 15890-15893.
[8] Y. Ha, J. Sim, Y. Lee, M. Suh, *Anal. Chem.* **2016**, *88*, 2563-2569.
[9] R. F. Coburn, *J. Appl. Physiol.* **2012**, *112*, 1949-1955.
[10] H. Kitagishi, S. Minegishi, A. Yumura, S. Negi, S. Taketani, Y. Amagase, Y. Mizukawa, T. Urushidani, Y. Sugiura, K. Kano, *J. Am. Chem. Soc.* **2016**, *138*, 5417-5425.
[11] a) B. W. Michel, A. R. Lippert, C. J. Chang, *J. Am. Chem. Soc.* **2012**, *134*, 15668-15671; b) L. Yuan, W. Lin, L. Tan, K. Zheng, W. Huang, *Angew. Chem. Int. Edit.* **2013**, *52*, 1628-1630; c) K. Zheng, W. Lin, L. Tan, H. Chen, H. Cui, *Chem. Sci.* **2014**, *5*, 3439-3448.
[12] J. S. Cannon, J. H. Frederich, L. E. Overman, *J. Org. Chem.* **2012**, *77*, 1939-1951.
[13] H. Chen, B. Dong, Y. Tang, W. Lin, *Acc. Chem. Res.* **2017**, *50*, 1410-1422.
[14] R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann, C. J Green, *Circ. Res.* **2002**, *90*, e17-e24..
[15] a) K. Chen, R. B. Cole, B. B. Rees, *J. Proteomics* **2013**, *78*, 477-485; b) P. A. Padilla, M. B. Roth, *P. Natl. Acad. Sci. USA* **2001**, *98*, 7331-7335.

10.1002/anie.201707518

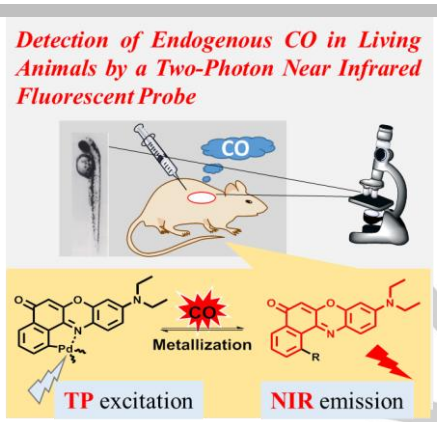

WILEY-VCH

**Entry for the Table of Contents**

Layout 1:

# COMMUNICATION

A unique Pd(II)-based two-photon excited near-infrared fluorescent probe (**1-Ac**) for endogenous CO was rationally designed and constructed. The robust probe exhibited superior properties, which allow for tracking of endogenous CO in zebrafish embryos and mouse tissues for the first time.




*Keyin Liu [§], Xiuqi Kong [§], Yanyan Ma, and Weiying Lin**

***Page No. – Page No.***

**Rational design of a robust fluorescent probe for detecting endogenous carbon monoxide in living zebrafish embryos and mouse tissues**