ACS Partner Journal

CHEMICAL & BIOMEDICAL IMAGING

Editors' Choice

Open Access

pubs.acs.org/ChemBioImaging

Viewpoint

Fluorogenic Tetrazine Bioorthogonal Probes for Advanced Application in Bioimaging and Biomedicine

Wuyu Mao, Ping Dong, Wei Du,* and Haoxing Wu*

Cite This: *Chem. Biomed. Imaging* 2025, 3, 1−4

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: A variety of bioorthogonal chemical tools have been developed and widely used in the study of biological phenomena in situ. Tetrazine bioorthogonal chemistry exhibits ultrafast reaction kinetics, excellent biocompatibility, and precise optical regulatory capabilities. Fluorogenic tetrazine bioorthogonal probes have achieved particularly diverse applications in bioimaging and disease diagnosis and treatment. This Viewpoint briefly introduces the characteristics and advantages of tetrazine bioorthogonal chemistry, some design strategies of fluorogenic tetrazine probes, and the status of applications of these tools to in vivo imaging, as well as disease diagnosis and treatment. Finally, we discuss challenges and propose future trends in the field of fluorogenic tetrazine probes. This Viewpoint offers insights into the development of new bioorthogonal tools for chemical biology research and for the design of new drugs.

First described by Carolyn Bertozzi in 1997,[1] "bioorthogonal chemistry" utilizes chemical reactions of non-native functional groups to explore biological processes without disrupting the function of native biomolecules. In addition to the use of these tools to study living organisms, the continuing development of highly efficient bioorthogonal reactions[2] has enabled the spatiotemporal control of proteins and epigenetic signals,[3] the directed activation of prodrugs,[4,5] and the specific targeting of compounds used for molecular imaging or radioimmunotherapy.[6−9]

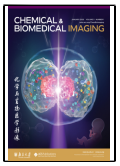
Reactions involving tetrazine have been found to exhibit the ultrafast kinetics and biocompatibility that are required for bioorthogonal chemistry. The rate constant of the reaction between tetrazine and the dienophile *trans*-cyclooctene (TCO) approaches 10^7 M^{-1} s^{-1}, suggesting the possibility of analyzing modified targets that are present below nanomolar concentrations.[2,10] Additionally, reaction involving tetrazine afford the possibility of targeted fluorescence imaging with low background emissions. This property involves a $n-\pi^*$ electron transition of tetrazine near 520 nm, which quenches the photoexcitation energy of adjacent fluorophores, decreasing undesired background fluorescence. Fluorescence turn-on is achieved within this dark environment upon a bioorthogonal reaction with specific targets that converts tetrazine into (dihydrogen)pyridazine, thereby revealing fluorescence with a high signal-to-noise ratio.[10,11] Due to these advantages, many recent efforts have focused on the development of fluorogenic tetrazine probes for a variety of biomedical applications (Figures 1 and 2).[10,12,13]

Efforts have been put forth to engineer tetrazine-based probes to improve the flexibility of their fluorogenic properties. For example, the development of probes with fluorescence in the near-infrared (NIR) range has taken advantage of the deep tissue penetration and low tissue autofluorescence associated with NIR radiation. NIR fluorogenic tetrazine probes exhibit a relatively high signal-to-noise ratio that has facilitated in vivo imaging in studies of metabolic changes during analyses of disease-related microenvironments and cancer-associated proteins.[14] NIR-sensitive probes have also been used clinically in the diagnosis, prognosis, and treatment of multiple diseases.

Manipulation of the fluorescence properties of tetrazine probes has also been applied to the development of bioorthogonally activatable phototherapy. Here, in addition to quenching fluorescence by disturbing the excited singlet state, tetrazine can also dampen the excited triplet state of a photosensitizer to reduce photodynamic efficiency. When applied to off-target tissues, this strategy can improve tumor targeting and thus treatment outcomes in addition to reducing toxic side effects. Thus, bioorthogonally activatable phototherapy represents a promising strategy for the precise targeted therapy of several types of cancer.[15]

Multiple strategies have been developed to apply the fluorogenic characteristics of tetrazine probes to the tracking of the dynamics of subcellular structures, the study of interactions among subcellular structures and cells, the quantitative analysis of biomolecules, and the monitoring of biological signaling within live cells. Combined with advanced imaging techniques such as stimulated emission depletion (STED) and total internal reflection fluorescence (TIRF), fluorogenic tetrazine probes have achieved high- and super-resolution and high-contrast imaging of mitochondria, lysosomes, lipid droplets, the endoplasmic reticulum, the

Received: November 30, 2024
Revised: December 5, 2024
Accepted: December 8, 2024
Published: December 23, 2024



https://doi.org/10.1021/cbmi.4c00095
Chem. Biomed. Imaging 2025, 3, 1−4

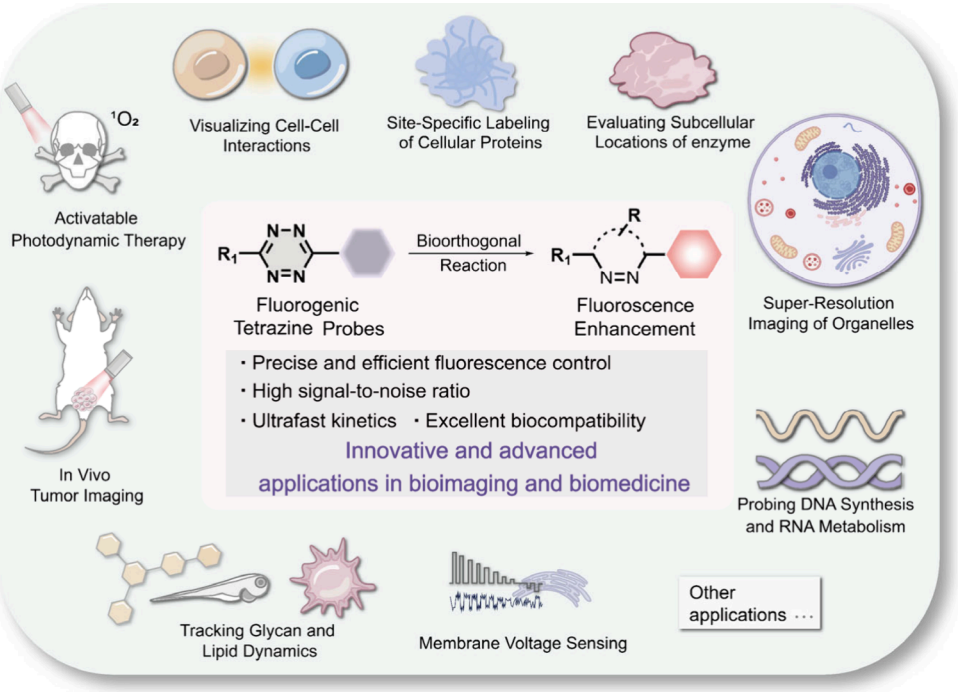


Figure 1. Representative applications of fluorogenic tetrazine bioorthogonal probes.

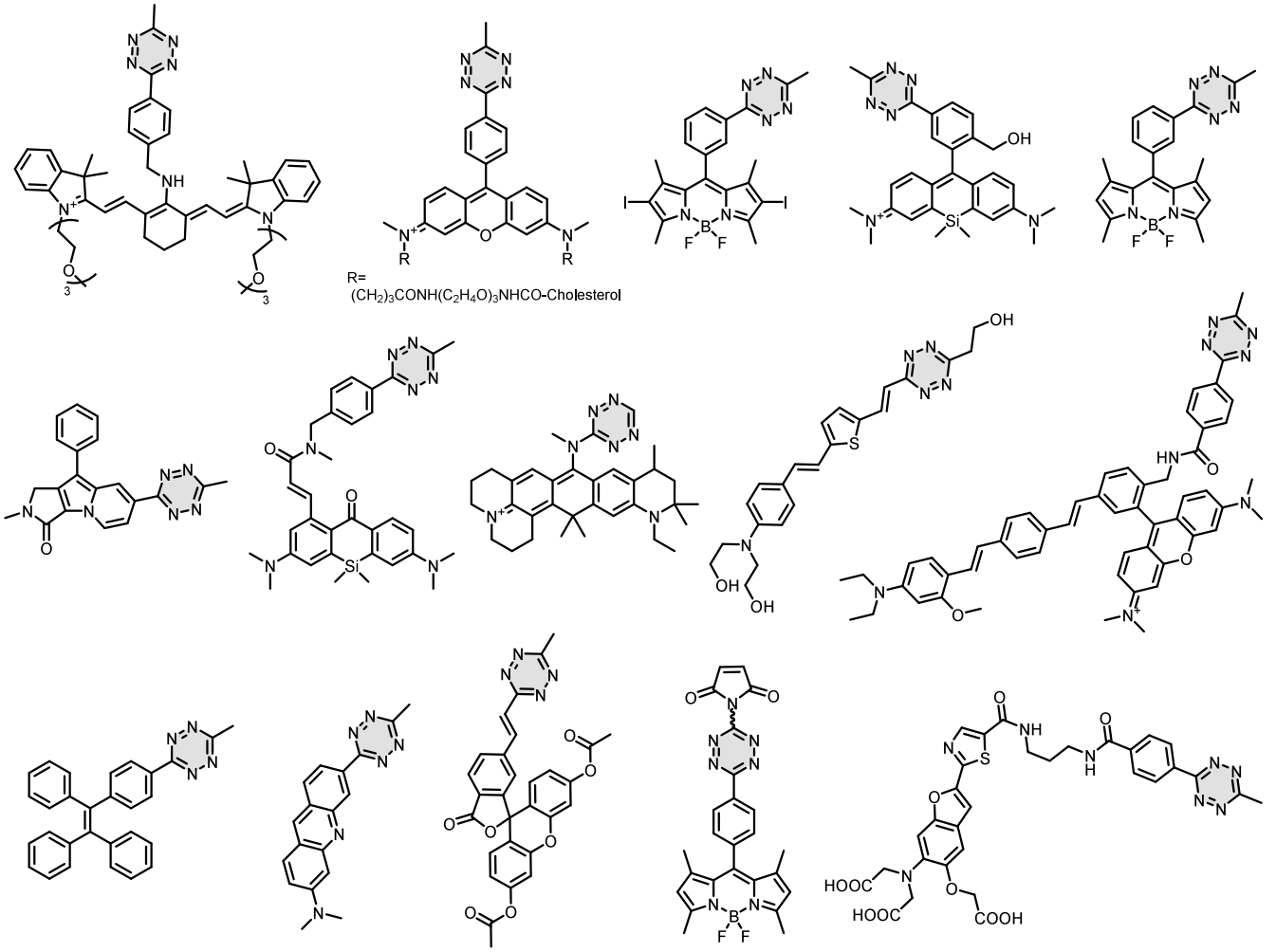


Figure 2. Chemical structures of representative fluorogenic tetrazine bioorthogonal probes.

https://doi.org/10.1021/cbmi.4c00095
Chem. Biomed. Imaging 2025, 3, 1−4

nucleus, the cytoskeleton, the Golgi apparatus, and other subcellular structures.[10,16−20] The design of tetrazine probes with mutually orthogonal reactivity toward different bioorthogonal partners has permitted advanced multiplex imaging, involving the simultaneous labeling and visualizing of the dynamics of multiple subcellular structures in live cells.[16]

Tetrazine probes have also been targeted to specific biomolecules. For example, to monitor DNA and RNA during mitotic cycles, a fluorogenic intercalating acridine-tetrazine probe was developed to react with metabolic vinyl deoxyuridine or uridine.[21] The design of a 2TCO*a*-modified 2′-deoxycytidine triphosphate reporter that was readily incorporated into DNA upon metabolic labeling, led to comprehensive fluorescence imaging of DNA in living cells, when combined with a fluorogenic tetrazine-coumarin partner probe.[22] To detect tumor-associated miRNA in live cells, a fluorogenic tetrazine-mediated transfer reaction with enhanced turnover has been developed, and it has been used to identify the target when present in picomole quantities.[23]

Tetrazine-based bioorthogonal chemistry techniques have also been applied to the protein visualization.[24] When combined with metabolic labeling that glycoprotein decorated with BCN dienophile, fluorogenic tetrazine−Oregon Green conjugates have been employed to monitor the changes in sialylated glycoconjugates during the development of zebrafish embryos.[25] To analyze the activation and dynamic subcellular localization of phospholipase D, a series of probes was designed to be utilized by this enzyme as a substrate in place of water. Metabolic labeling with these TCO-containing alcohols followed by tetrazine-BODIPY probe staining led to detailed maps of enzyme action within cells upon introduction of a variety of drugs.[26] Fluorogenic tetrazine probes have also been designed to allow imaging studies of multiple other aspects of cells, including the potentials of the plasma membrane and organellar membranes,[18,27] lipid metabolism,[28] pathology-related microenvironmental changes,[29] and cell interactions.[30] These efforts have led to important advances in related research fields.

Significant progress has already been achieved in the design of fluorogenic tetrazine probes, but many challenges remain. For instance, a general tetrazine quenching strategy that can be applied to all dyes has not yet been developed; notably, the physicochemical mechanisms leading to quenching are not fully understood, and current tetrazines are limited in the ways that they quench NIR dyes. The rational design of fluorogenic tetrazine probes using computational chemistry, including artificial intelligence and machine learning, is necessary in order to improve the quenching of classical fluorophores and to facilitate novel dye design strategies. In addition, fluorogenic probes with deeper tissue penetration are required in order to take advantage of the penetrative properties of NIR-II light and to more effectively monitor changes in whole organisms. Finally, as new tetrazine probes are developed, the targetability and biocompatibility should be comprehensively considered to expand their application range, especially considering the potential power of dual-targeting bioorthogonal systems of tetrazine and dienophile probes in the simultaneous and synergistic study of multiple targets. In summary, proposing new mechanisms and developing new fluorogenic tetrazine probes promises to provide indispensable tools for life science research and more options for precise diagnosis and treatment of diseases.

AUTHOR INFORMATION

Corresponding Authors

Wei Du − *Key Laboratory of Drug-Targeting and Drug Delivery System of the Education Ministry and Sichuan Province, and Sichuan Research Center for Drug Precision Industrial Technology, West China School of Pharmacy, Sichuan University, Chengdu 610041, China;* orcid.org/0000-0003-4162-1471; Email: duweiyb@scu.edu.cn

Haoxing Wu − *Department of Radiology, Huaxi MR Research Center (HMRRC), Institution of Radiology and Medical Imaging and Functional and Molecular Imaging Key Laboratory of Sichuan Province, West China Hospital of Sichuan University, Chengdu 610041 Sichuan, China;* orcid.org/0000-0002-0008-4668; Email: haoxingwu@scu.edu.cn

Authors

Wuyu Mao − *Department of Respiratory and Critical Care Medicine, Institute of Respiratory Health, Frontiers Science Center for Disease-Related Molecular Network, State Key Laboratory of Respiratory Health and Multimorbidity, West China Hospital of Sichuan University, Chengdu 610041, China;* orcid.org/0000-0002-6143-5943

Ping Dong − *Department of Nuclear Medicine, West China Hospital, Sichuan University, Chengdu 610041, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/cbmi.4c00095

Author Contributions

All authors contributed to the paper writing, discussion, and editing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful for the financial support from the National Natural Science Foundation of China (No. 22277083), Natural Science Foundation of Sichuan Province (Grant Nos. 2023NSFSC5333 2024NSFTD0023).

REFERENCES

(1) Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. Engineering Chemical Reactivity on Cell Surfaces Through Oligosaccharide Biosynthesis. *Science* **1997**, *276*, 1125−1128.

(2) Scinto, S. L.; Bilodeau, D. A.; Hincapie, R.; Lee, W.; Nguyen, S. S.; Xu, M.; am Ende, C. W.; Finn, M. G.; Lang, K.; Lin, Q. Bioorthogonal chemistry. *Nat. Rev. Methods Primers* **2021**, *1*, 30.

(3) Zhu, Y.; Ding, W.; Chen, Y.; Shan, Y.; Liu, C.; Fan, X.; Lin, S.; Chen, P. R. Genetically encoded bioorthogonal tryptophan decaging in living cells. *Nat. Chem.* **2024**, *16*, 533−542.

(4) He, X.; Li, J.; Liang, X.; Mao, W.; Deng, X.; Qin, M.; Su, H.; Wu, H. An all-in-one tetrazine reagent for cysteine-selective labeling and bioorthogonal activable prodrug construction. *Nat. Commun.* **2024**, *15*, 2831.

(5) Wang, J.; Wang, X.; Fan, X.; Chen, P. R. Unleashing the Power of Bond Cleavage Chemistry in Living Systems. *ACS Cent. Sci.* **2021**, *7*, 929−943.

(6) Fu, Q.; Shen, S.; Sun, P.; Gu, Z.; Bai, Y.; Wang, X.; Liu, Z. Bioorthogonal chemistry for prodrug activation in vivo. *Chem. Soc. Rev.* **2023**, *52*, 7737−7772.

(7) Weng, J.; Huang, Z.; Liu, Y.; Wen, X.; Miao, Y.; Xu, J.-J.; Ye, D. Controlled In Situ Self-Assembly of Biotinylated Trans-Cyclooctene Nanoparticles for Orthogonal Dual-Pretargeted Near-Infrared Fluo-

rescence and Magnetic Resonance Imaging. *J. Am. Chem. Soc.* **2024**, *146*, 13163−13175.
(8) Bauer, D.; Sarrett, S. M.; Lewis, J. S.; Zeglis, B. M. Click chemistry: a transformative technology in nuclear medicine. *Nat. Protoc.* **2023**, *18*, 1659−1668.
(9) Yang, J.; Zhu, B.; Ran, C. The Application of Bio-orthogonality for In Vivo Animal Imaging. *Chem. Biomed. Imaging* **2023**, *1*, 434−447.
(10) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse electron demand Diels-Alder reactions in chemical biology. *Chem. Soc. Rev.* **2017**, *46*, 4895−4950.
(11) Sun, H.; Xue, Q.; Zhang, C.; Wu, H.; Feng, P. Derivatization based on tetrazine scaffolds: synthesis of tetrazine derivatives and their biomedical applications. *Org. Chem. Front.* **2022**, *9*, 481−498.
(12) Zhao, X.; Du, J.; Sun, W.; Fan, J.; Peng, X. Regulating Charge Transfer in Cyanine Dyes: A Universal Methodology for Enhancing Cancer Phototherapeutic Efficacy. *Acc. Chem. Res.* **2024**, *57*, 2582−2593.
(13) Chen, Y.; Jiang, H.; Hao, T.; Zhang, N.; Li, M.; Wang, X.; Wang, X.; Wei, W.; Zhao, J. Fluorogenic Reactions in Chemical Biology: Seeing Chemistry in Cells. *Chem. Biomed. Imaging* **2023**, *1*, 590−619.
(14) Zhang, X.; Gao, J.; Tang, Y.; Yu, J.; Liew, S. S.; Qiao, C.; Cao, Y.; Liu, G.; Fan, H.; Xia, Y.; et al. Bioorthogonally activatable cyanine dye with torsion-induced disaggregation for in vivo tumor imaging. *Nat. Commun.* **2022**, *13*, 3513.
(15) Kozma, E.; Bojtar, M.; Kele, P. Bioorthogonally Assisted Phototherapy: Recent Advances and Prospects. *Angew. Chem., Int. Ed.* **2023**, *62*, No. e202303198.
(16) Deng, Y.; Shen, T.; Yu, X.; Li, J.; Zou, P.; Gong, Q.; Zheng, Y.; Sun, H.; Liu, X.; Wu, H. Tetrazine-Isonitrile Bioorthogonal Fluorogenic Reactions Enable Multiplex Labeling and Wash-Free Bioimaging of Live Cells. *Angew. Chem., Int. Ed.* **2024**, *63*, No. e202319853.
(17) Aktalay, A.; Lincoln, R.; Heynck, L.; Lima, M.; Butkevich, A. N.; Bossi, M. L.; Hell, S. W. Bioorthogonal Caging-Group-Free Photoactivatable Probes for Minimal-Linkage-Error Nanoscopy. *ACS Cent. Sci.* **2023**, *9*, 1581−1590.
(18) Klier, P. E. Z.; Gest, A. M. M.; Martin, J. G.; Roo, R.; Navarro, M. X.; Lesiak, L.; Deal, P. E.; Dadina, N.; Tyson, J.; Schepartz, A.; Miller, E. W. Bioorthogonal, Fluorogenic Targeting of Voltage-Sensitive Fluorophores for Visualizing Membrane Potential Dynamics in Cellular Organelles. *J. Am. Chem. Soc.* **2022**, *144*, 12138−12146.
(19) Segawa, S.; Wu, J.; Kwok, R. T. K.; Wong, T. T. W.; He, X.; Tang, B. Z. Co-aggregation as A Simple Strategy for Preparing Fluorogenic Tetrazine Probes with On-Demand Fluorogen Selection. *Angew. Chem., Int. Ed.* **2024**, *63*, No. e202313930.
(20) Kim, D.; Son, H.; Park, S. B. Ultrafluorogenic Monochromophore-Type BODIPY-Tetrazine Series for Dual-Color Bioorthogonal Imaging with a Single Probe. *Angew. Chem., Int. Ed.* **2023**, *62*, No. e202310665.
(21) Loehr, M. O.; Luedtke, N. W. A Kinetic and Fluorogenic Enhancement Strategy for Labeling of Nucleic Acids. *Angew. Chem., Int. Ed.* **2022**, *61*, No. e202112931.
(22) Sterrenberg, V. T.; Stalling, D.; Knaack, J. I. H.; Soh, T. K.; Bosse, J. B.; Meier, C. A TriPPPro-Nucleotide Reporter with Optimized Cell-Permeable Dyes for Metabolic Labeling of Cellular and Viral DNA in Living Cells. *Angew. Chem., Int. Ed.* **2023**, *62*, No. e202308271.
(23) Wu, H.; Cisneros, B. T.; Cole, C. M.; Devaraj, N. K. Bioorthogonal tetrazine-mediated transfer reactions facilitate reaction turnover in nucleic acid-templated detection of microRNA. *J. Am. Chem. Soc.* **2014**, *136*, 17942−17945.
(24) Hao, M.; Ling, X.; Sun, Y.; Wang, X.; Li, W.; Chang, L.; Zeng, Z.; Shi, X.; Niu, M.; Chen, L.; Liu, T. Tracking endogenous proteins based on RNA editing-mediated genetic code expansion. *Nat. Chem. Biol.* **2024**, *20*, 721−731.
(25) Agarwal, P.; Beahm, B. J.; Shieh, P.; Bertozzi, C. R. Systemic Fluorescence Imaging of Zebrafish Glycans with Bioorthogonal Chemistry. *Angew. Chem., Int. Ed.* **2015**, *54*, 11504−11510.
(26) Liang, D.; Wu, K.; Tei, R.; Bumpus, T. W.; Ye, J.; Baskin, J. M. A real-time, click chemistry imaging approach reveals stimulus-specific subcellular locations of phospholipase D activity. *Proc. Natl. Acad. Sci. U S A* **2019**, *116*, 15453−15462.
(27) Liu, S.; Lin, C.; Xu, Y.; Luo, H.; Peng, L.; Zeng, X.; Zheng, H.; Chen, P. R.; Zou, P. A far-red hybrid voltage indicator enabled by bioorthogonal engineering of rhodopsin on live neurons. *Nat. Chem.* **2021**, *13*, 472−479.
(28) Bertheussen, K.; van de Plassche, M.; Bakkum, T.; Gagestein, B.; Ttofi, I.; Sarris, A. J. C.; Overkleeft, H. S.; van der Stelt, M.; van Kasteren, S. I. Live-Cell Imaging of Sterculic Acid—a Naturally Occurring 1,2-Cyclopropene Fatty Acid—by Bioorthogonal Reaction with Turn-On Tetrazine-Fluorophore Conjugates. *Angew. Chem., Int. Ed.* **2022**, *61*, e202207640.
(29) Zhou, L.; Wang, Z.; Wang, L.; Zhang, X.; Xiao, Y. Tetrazine-Based Ratiometric Nitric Oxide Sensor Identifies Endogenous Nitric Oxide in Atherosclerosis Plaques by Riding Macrophages as a Smart Vehicle. *J. Am. Chem. Soc.* **2023**, *145*, 28296−28306.
(30) Xue, E. Y.; Lee, A. C. K.; Chow, K. T.; Ng, D. K. P. Promotion and Detection of Cell−Cell Interactions through a Bioorthogonal Approach. *J. Am. Chem. Soc.* **2024**, *146*, 17334−17347.

https://doi.org/10.1021/cbmi.4c00095
Chem. Biomed. Imaging 2025, 3, 1−4