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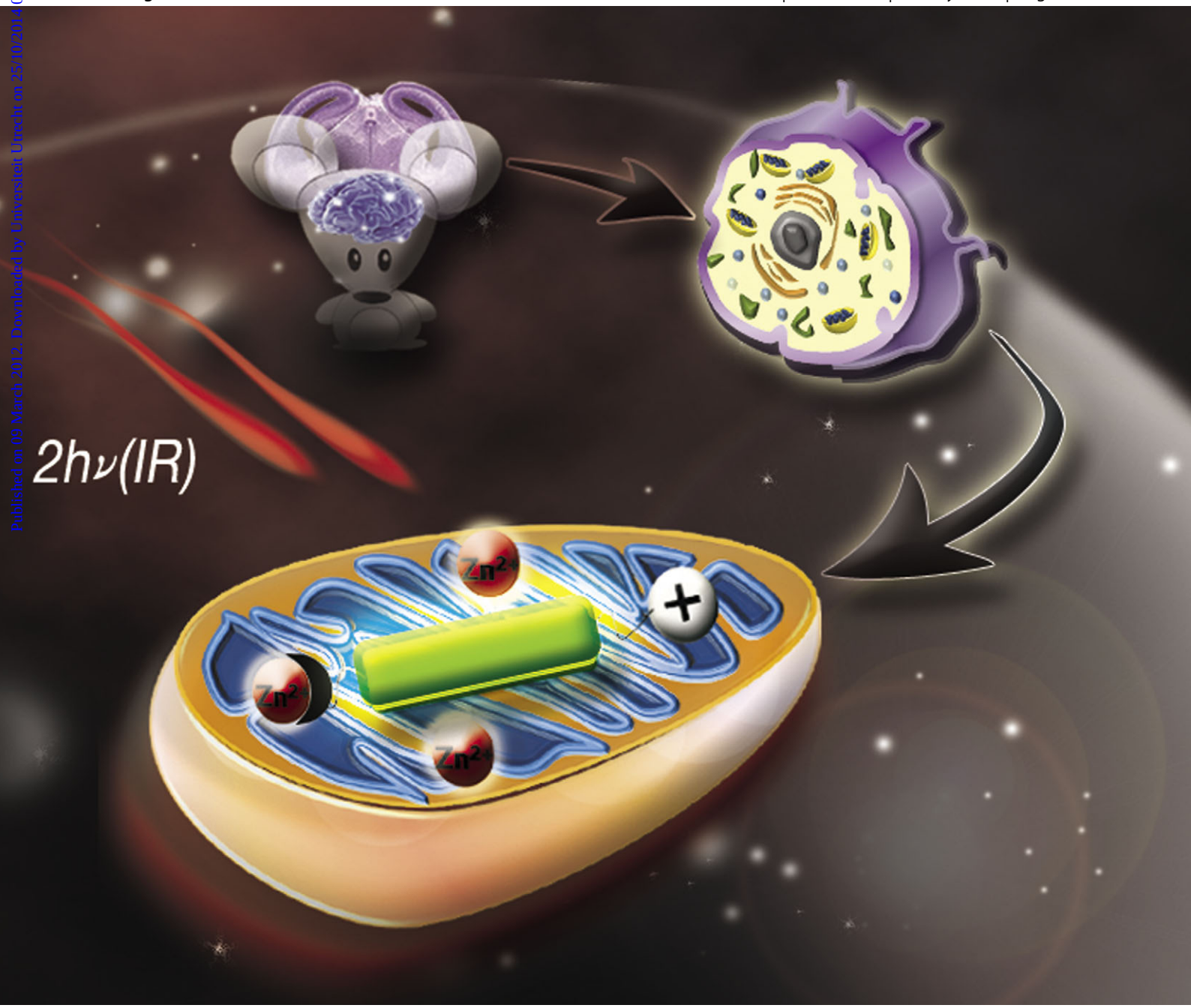

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A highly sensitive two-photon fluorescent probe for mitochondrial zinc ions in living tissue†

Na Young Baek,‡[a] Cheol Ho Heo,‡[a] Chang Su Lim,[b] Goutam Masanta,[a] Bong Rae Cho*[b] and Hwan Myung Kim*[a]

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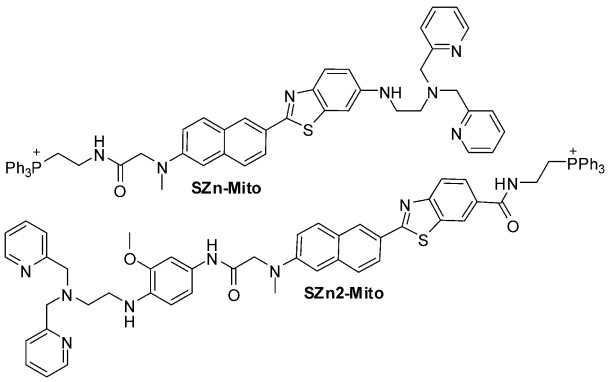
We report a highly sensitive two-photon probe (SZn2-Mito) which shows a 70-fold two-photon excited fluorescence enhancement in response to Zn^{2+} and can selectively detect mitochondrial Zn^{2+} in a rat hippocampal slice at a depth of 100–200 μm by using two-photon microscopy.

The zinc ion is the second-most-abundant d-block metal ion in the human brain and is an active component in enzymes and proteins.[1,2] For proper brain functions, it is vital to maintain Zn^{2+}-ion homeostasis, which is controlled by the import of intracellular Zn^{2+} ions ($[Zn^{2+}]_i$) from—and export to—the extracellular space, the endoplasmic reticulum, and mitochondria. While mitochondria can take up evoked $[Zn^{2+}]_i$ rise,[3,4] a strong elevation of intra-mitochondrial Zn^{2+} ($[Zn^{2+}]_m$) can promote mitochondrial dysfunctions.[3,4] To understand the physiology of Zn^{2+} in the brain, it is crucial to monitor $[Zn^{2+}]_m$ in intact brain tissue. An attractive approach to the detection of $[Zn^{2+}]_m$ is the use of two-photon microscopy (TPM). TPM, which employs two near-infrared photons as the excitation source, is a new technique that can visualize biological events deep inside intact tissues (>500 μm) for extended periods of time when combined with an appropriate two-photon (TP) probe.[5,6] Recently, we reported a targetable TP probe (SZn-Mito) that has been successfully utilized to detect $[Zn^{2+}]_m$ in live cells and tissues by TPM.[7] However, this initial design was limited by the modest turn-on response (7-fold) in the presence of excess Zn^{2+}. For more sensitive detection, there is a need to develop a highly sensitive TP probe for $[Zn^{2+}]_m$.

With this aim in mind, we have designed a new TP probe for $[Zn^{2+}]_m$ (SZn2-Mito, Scheme 1) derived from 6-(benzo[*d*]thiazol-2′-yl)-2-(*N*,*N*-dimethylamino)naphthalene (BTDAN) as the reporter, a methoxy derivative of *N*,*N*-di-(2-picolyl)ethylenediamine (MeO-DPEN) as the Zn^{2+} chelator, and triphenylphosphonium salt (TPP) as the mitochondrial-targeting site (Scheme 1).[8–10] MeO-DPEN has been employed as the receptor because it has been shown to increase the Zn^{2+} binding affinity and turn-on response.[11] Herein, we report that SZn2-Mito is a much more sensitive TP probe, and can detect $[Zn^{2+}]_m$ in live cells and intact tissues at >100 μm depth with brighter and clearer images by TPM when compared with SZn-Mito.



Scheme 1 Structures of SZn-Mito and SZn2-Mito.

The preparation of SZn2-Mito is described in the ESI.† The solubility of SZn2-Mito in buffer solution (50 mM HEPES, 100 mM KCl, pH 7.4) is approximately 2 μM, which is sufficient to stain the cells (Fig. S2 in the ESI†). The absorption and emission spectra of SZn2-Mito showed gradual red-shifts with increasing solvent polarity. The shifts of emission spectra were greater for SZn2-Mito than for SZn-Mito ($\Delta\lambda_{fl}$ = 80 *vs.* 45 nm).[7] The larger Stokes shift observed in SZn2-Mito compared to SZn-Mito is likely to be due to greater stabilization of the charge transfer excited state of the former fluorophore that contains a stronger electron-withdrawing group (Fig. S1 and Table S1 in ESI†).

In metal free buffer (50 mM HEPES, 100 mM KCl, 10 mM NTA, pH 7.4), SZn2-Mito has the absorption (λ_{max}) and emission maxima (λ^{fl}_{max}) at 413 nm ($\varepsilon = 1.85 \times 10^4$ M^{-1} cm^{-1}) and 536 nm (Φ = 0.0048), respectively (Table S2 in ESI†). Upon addition of Zn^{2+}, the fluorescence intensity increased dramatically with a slight blue shift in its absorption spectra (Fig. S3a,b in ESI†), presumably due to the blocking of the photoinduced electron transfer (PeT) process upon complexation with Zn^{2+}. A nearly identical result was observed in the TP process (Fig. 1a). The fluorescence enhancement factor [FEF = $(F - F_{min})/F_{min}$] of SZn2-Mito determined for the

[a] *Division of Energy Systems Research and Molecular Science & Technology Research Center, Ajou University, Suwon, 443-749, Korea. E-mail: kimhm@ajou.ac.kr; Fax: +82-31-219-1615*
[b] *Department of Chemistry, Korea University, 145, Anamro, Sungbuk-gu, Seoul, 136-713, Korea. E-mail: chobr@korea.ac.kr; Fax: +82-2-3290-3544*

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‡ These two authors contributed equally to this work.

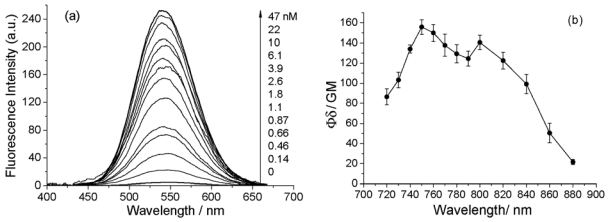

Fig. 1 (a) Two-photon fluorescence spectra of 1 μM SZn2-Mito in the presence of free Zn^{2+} (0–47 nM). The excitation wavelength is 750 nm with fs pulse. (b) Two-photon action spectra of SZn2-Mito in the presence of 47 nM free Zn^{2+}. These data were measured in 50 mM HEPES buffer (100 mM KCl, 10 mM NTA, pH 7.4).

one-photon process was approximately 70 in the presence of excess Zn^{2+} (Table S2, ESI†); the FEF of SZn2-Mito is 10-fold larger than that of SZn-Mito.[7] The much larger turn-on response determined for SZn2-Mito can be attributed to its much smaller fluorescence quantum yield (Table S2 in ESI†). This result underlines the importance of stabilizing the charge transfer excited state for the design of an efficient fluorescent probe with a large turn-on response.

The dissociation constants (K_d^{OP} and K_d^{TP}) of SZn2-Mito for the one- and two-photon processes were determined from the fluorescence titration curves (Fig. S3c,d, ESI†).[12] The titration curves fitted well with a 1 : 1 binding model (Fig. S3c, ESI†), the Hill plots were linear with a slope of 1.0 (Fig. S3d, ESI†), and the Job's plot exhibited a maximum point at the mole fraction 0.50 (Fig. S4, ESI†), indicating 1 : 1 complexation between SZn2-Mito and Zn^{2+}. The K_d^{OP} and K_d^{TP} values for the SZn2-Mito/Zn^{2+} complex were 1.4 nM, which was 2.2-fold smaller than that for SZn-Mito (3.1 nM).[7] The stronger Zn^{2+} binding affinity of MeO-DPEN than DPEN was previously reported.[11] Moreover, SZn2-Mito exhibits high selectivity for 1 μM Zn^{2+} compared with 1 mM of Na^+, K^+, Ca^{2+}, Mg^{2+}, 1 μM of Mn^{2+}, Fe^{2+}, Co^{2+}, Pb^{2+} and modest selectivity over 1 μM Ni^{2+}, Cd^{2+} (Fig. S5b, ESI†). In the presence of 1 μM of Cu^{2+} and Hg^{2+}, the fluorescence was quenched due to metal-to-ligand electron transfer upon excitation.[13] Since Ni^{2+}, Cd^{2+}, Hg^{2+} and Cu^{2+} ions rarely exist in the cells,[14] this probe can detect Zn^{2+} with minimum interference from other competing metal ions. Further, SZn2-Mito is pH-insensitive in the biologically relevant pH range (Fig. S5a, ESI†). The combined results establish that SZn2-Mito can serve as a highly sensitive turn-on probe for detecting Zn^{2+} with minimum interference from other competing metal ions and pH.

We also evaluated the ability of SZn2-Mito to detect Zn^{2+} in a TP mode. The TP action spectrum of SZn2-Mito in metal free HEPES buffer was too small to measure accurately. In the presence of excess Zn^{2+}, however, the $\Phi\delta$ value increased dramatically to 155 GM at 750 nm (Fig. 1b and Table S2 in ESI†). Moreover, the $\Phi\delta$ value is larger by 2-fold than 75 GM for SZn-Mito, a result that can be attributed to the enhanced intramolecular charge transfer (see above).[15] This outcome predicts twice as bright TPM images of the cells and tissues labeled with SZn2-Mito as those labeled with SZn-Mito (see below).

We next sought to utilize SZn2-Mito to detect $[Zn^{2+}]_m$ in a cellular environment. For this experiment, HeLa cells were co-labeled with SZn2-Mito and Mitotracker Red FM,

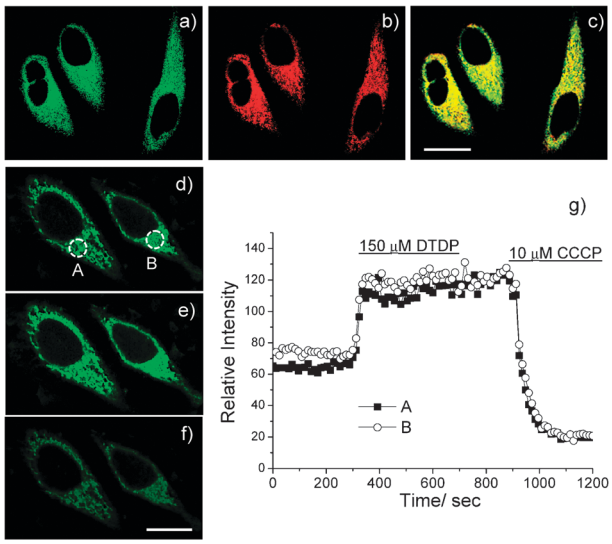

Fig. 2 (a) TPM and (b) OPM images of HeLa cells co-labeled with (a) SZn2-Mito (1 μM) and (b) Mitotracker Red FM (1 μM). (c) Colocalized image. The wavelengths for one- and two-photon excitation were 514 and 750 nm, respectively, and the emission was collected at 450–550 (SZn-Mito) and 600–700 nm (Mitotracker Red FM), respectively. Scale bar, 20 μm. (d–f) TPM images of 1 μM SZn2-Mito-labeled HeLa cells, before (d) and after (e) addition of 150 μM DTDP to the imaging solution. (f) After addition of 10 μM CCCP to (e). (g) The relative TPEF intensity of SZn2-Mito-labeled HeLa cells as a function of time. The TPEF intensities were collected at 450–600 nm upon excitation at 750 nm with fs pulse. Scale bar, 10 μm. Cells shown are representative images from replicate experiments (n = 5).

a well-known OP fluorescent probe for mitochondria.[16] The TPM and OPM images were obtained by using the detection windows at 450–550 and 600–700 nm, respectively, to selectively detect the two signals with equal intensities (Fig. S7, ESI†). The two images overlapped well (Fig. 2a–c), and Pearson's colocalization coefficient, A, calculated by using Autoquant X2 software, of SZn2-Mito with Mitotracker Red FM was 0.85.[17] Moreover, the TPEF intensity of SZn2-Mito-labeled HeLa cells decreased dramatically upon addition of *N,N,N′,N′*-tetrakis(2-pyridyl)ethylenediamine (TPEN), a membrane permeable Zn^{2+} chelator that can effectively remove $[Zn^{2+}]_m$ (Fig. S6a,b, ESI†).[3b] These results confirm that the bright regions in the TPM image reflect $[Zn^{2+}]_m$. Further, SZn2-Mito showed negligible toxicity as measured by using a CCK-8 kit (Fig. S8, ESI†), and high photostability as indicated by the negligible decay in the TPEF intensity at a given spot on the SZn2-Mito-labeled HeLa cells after continuous irradiation of the fs-pulses for 60 min (Fig. S6d, ESI†).

We then tested whether SZn2-Mito can monitor the changes in $[Zn^{2+}]_m$ in live cells. Upon TP excitation at 750 nm, the TPEF intensity increased dramatically when 2,2′-dithiodipyridine (DTDP; 150 μM), a reagent that promotes the release of Zn^{2+} from Zn^{2+}-binding proteins,[18] was added to HeLa cells labeled with SZn2-Mito (Fig. 2d,e,g), and decreased abruptly upon addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 10 μM, Fig. 2f,g), a compound that promotes the release of intramitochondrial cations by collapsing the mitochondrial membrane potential.[19] The TPEF intensity also increased when the cells were treated with 100 μM Zn^{2+} and

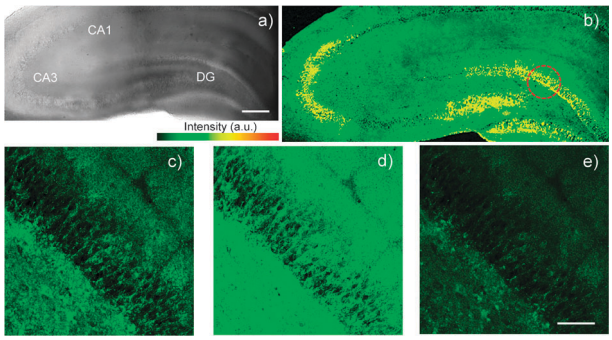

Fig. 3 Images of a rat hippocampal slice stained with 20 μM SZn2-Mito for 1 h. (a) Bright-field images of the CA1–CA3 regions as well as dentate gyrus (DG) at 10× magnification. (b) 10 TPM images along the *z*-direction at the depths of approximately 100–200 μm were accumulated. (c–e) Magnification at 40× in the DG regions (red circle in (b)) at a depth of ~100 μm (c) before and (d) after addition of 150 μM DTDP to the imaging solution. (e) After addition of 10 μM CCCP to (d). Scale bars: (a) 300 and (e) 75 μm.

100 μM pyrithione (2-mercaptopyridine *N*-oxide), a reagent that can bring Zn^{2+} into the cytoplasm,[20] and decreased by the treatment with CCCP (Fig. S9, ESI†). In all cases, the changes in TPEF intensity were larger than those observed with SZn-Mito. These results establish that SZn2-Mito can detect $[Zn^{2+}]_m$ in live cells with higher sensitivity than SZn-Mito.

We further investigated the utility of SZn2-Mito in tissue imaging. The TPM images were obtained from a slice of a 14 day-old rat hippocampal tissue incubated with 20 μM SZn2-Mito for 1 h at 37 °C. The slice from the brain was too large to show in one image, so two images were obtained in each *xy* plane and combined. The bright-field image revealed the CA1 and CA3 regions and also the dentate gyrus (DG; Fig. 3a). As the structure of the brain tissue is known to be inhomogeneous in its entire depth, we accumulated 10 TPM images at depths of 100–200 μm to visualize the distributions of the $[Zn^{2+}]_m$ (Fig. 3b). They reveal marked TPEF in the CA3 and DG regions (Fig. 3b).[11] At a higher magnification, the $[Zn^{2+}]_m$ distribution in the DG region is clearly visualized (Fig. 3c). As observed in the HeLa cells, the TPEF intensity increased after addition of DTDP and decreased upon treatment of CCCP, thereby providing a strong evidence that the bright regions reflect the $[Zn^{2+}]_m$ (Fig. 3d,e). Moreover, the TPM images obtained at a depth of 100–200 μm revealed the $[Zn^{2+}]_m$ distribution in the given plane along the *z* direction in the DG region (Fig. S10, ESI†). Hence, SZn2-Mito is clearly capable of detecting $[Zn^{2+}]_m$ at a depth of 100–200 μm in living tissues by using TPM. Further, the TPM images shown in Fig. 3b,c are brighter and clearer than those stained with SZn-Mito (Fig. S11, ESI†),[7] demonstrating the advantage of SZn2-Mito.

In conclusion, we have developed a mitochondrial-targeted and highly sensitive TP probe (SZn2-Mito) that shows a 70-fold TPEF enhancement in response to Zn^{2+}, a maximum TP action cross section value of 155 GM in the presence of excess Zn^{2+}, a dissociation constant (K_d^{TP}) of 1.4 nM, and pH insensitivity in the biologically relevant range. Compared with SZn-Mito, this probe shows 10-fold larger turn-on response, 2-fold larger $\Phi\delta$ value, and 2-fold stronger Zn^{2+} binding affinity, and can selectively detect $[Zn^{2+}]_m$ in live cells and tissues at a depth of 100–200 μm with a brighter and clearer TPM image.

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