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A Golgi-localized two-photon probe for imaging zinc ions

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We report a two-photon fluorescent probe which shows a strong two photon excited fluorescence enhancement in response to Zn\(^{2+}\), easy loading into the cells, Golgi-localizing ability, low cytotoxicity, and high photostability. Two-photon microscopy imaging revealed that this probe allows for real-time monitoring of the changes of Golgi Zn\(^{2+}\) as well as their 3D distributions in live cells and tissues.

Among the cell organelles, Golgi apparatus is a central station of secretion and transport of biomolecules. This organelle receives a variety of proteins and lipids from the endoplasmic reticulum (ER), and these cargoes are properly sorted into specific carriers for delivery to intra- and extra-cellular destinations.\(^1\) The defect of Golgi-related functions is closely related to many human disorders such as cancer and neuronal, kidney, liver, and eye diseases.\(^2\) The Golgi apparatus contains numerous resident proteins including metalloproteases and alkaline phosphatases whose catalytic activities mainly depend upon the Zn\(^{2+}\) ions. Zn\(^{2+}\) ions are also required for the structural stability of a variety of proteins involved in transcription and protein trafficking.\(^3\) Moreover, the Golgi apparatus plays an crucial role in maintaining the zinc homeostasis by sequestering excess Zn\(^{2+}\) ions and hence maintain the proper functions of the cell.\(^4\) Therefore, a method for direct visualization of Zn\(^{2+}\) ions in the Golgi apparatus ([Zn\(^{2+}\)]\(_{\text{Golgi}}\)) would help elucidate the Golgi-related physiological and pathological processes.

Organelle-selective fluorescent probes offer a direct imaging tool for studying the morphological activities of the organelles as well as the local distribution of biomolecules.\(^5\) To target lysosomes and mitochondria, various small molecule probes have been developed by attaching a well-characterized targeting moiety such as a tertiary amine and a lipophilic cation.\(^6\) However, the development of Golgi-localized small-molecule probes has remained a challenge. Also, although numerous fluorescent probes for detecting Zn\(^{2+}\) ions have been developed,\(^7\) the report on Golgi-localizable probe are very limited.\(^8\) Recently, transfection-based fluorescent probes for imaging [Zn\(^{2+}\)]\(_{\text{Golgi}}\) have been reported.\(^9\) However, this approach can vary in different cell types and is often technically difficult to apply in living tissues and animals. Further, these probes required short excitation wavelenths (< 600 nm) that can cause phototoxicity and artifactual generation of reactive oxygen species.\(^10\) Therefore, it is important to develop a small-molecule fluorescent probe that can selectively detect the [Zn\(^{2+}\)]\(_{\text{Golgi}}\) using two-photon microscopy (TPM). TPM utilizes longer wavelength excitation source (> 700 nm) that offers intrinsically high resolution with negligible background signal, reduced phototoxicity and photobleaching, and deeper tissue penetration depth (> 500 \(\mu\)m).\(^11\) Recently, various two-photon (TP) probes for detecting target analytes such as bio-metals,\(^12\) organelles,\(^13\) enzymes,\(^14\) and red-ox species\(^15\) have been developed. However, Golgi-localizable TP probes for detecting metal ions have never been exploited. We now report a TP fluorescent probe that can selectively detect [Zn\(^{2+}\)]\(_{\text{Golgi}}\) in live cells and tissues (SZnC, Scheme 1).

We prepared a TP probe for Zn\(^{2+}\) ions (SZn) by utilizing our previous strategy;\(^16\) BTDAN, 6-(benzo[d]thiazol-2'-yl)-2-([N,N-di-(2-picolyl)ethylenediamine (MeO -DPEN) as the TP fluorophore was coupled with a methoxy substituted N,N-di-(2-picolyl)ethylenediamine (MeO-DPEN) naphthalene, as the TP fluorophore was coupled with a methoxy substituted N,N-di-(2-picolyl)ethylenediamine (MeO-DPEN) as the Zn\(^{2+}\) chelator using a glycimamide spacer (Scheme 1). Similar strategy was used to design SZnC, excepting that a terminal carboxylic group (-COOH) as a solubility enhancing group was introduced. We also employed compound 1 as a counterpart to investigate the cellular localization. The preparation of SZn, SZnC and 1 is described in the supporting information.

![Scheme 1 Structures of SZn, SZnC and 1.](image-url)

In metal-free MOPS buffer (30 mM MOPS,100 mM KCl, 10 mM EGTA, pH = 7.2), SZn and SZnC exhibited absorption maxima (\(\lambda_{\text{abs}}\))...
at 389 nm and 384 nm with emission maxima (λ_{em}) at 497 nm (Φ = 0.02) and 518 nm (Φ = 0.08), respectively (Figure S1 and S2 Supporting Information). Upon the addition of Zn^{2+} ions (127 nM), dramatic 100- and 3-fold increases in the fluorescence intensities were observed by SZn and SZnC, respectively, with minimal changes in the absorption spectra (Figure S1, Supporting Information). These increases in emission intensity may be attributed to the blocking of the photo-induced electron transfer (PeT) process.\(^{17}\) The higher turn-on response of SZn may be attributed to the more efficient PeT from MeO-OPEN to BTBDAN than that for carboxyl containing SZnC. Moreover, the λ_{em} of both probes showed significant bathochromic shifts as the polarity of the solvent increased, on the order 1,4-dioxane < EtOH < EtOH:buffer (1:1) < buffer (Figure 1b,d). This characteristic allowed us to estimate the polarity of the probe microenvironment in the cells (see below).

We then tested the cell permeability of the probes. The TPM images of SZn-labeled live HeLa cells showed bright fluorescence throughout the entire cell excepting the nucleus region (Figure 1a). Interestingly, the images of SZnC-labeled cells revealed the tightly localized emission near the nucleus (Figure 1c), which might be the Golgi apparatus. These results indicated not only the good permeability of both probes but also the different cellular locations with the additional functional group. Further, the TP excited fluorescence (TPEF) spectra of SZn and SZnC measured in HeLa cells had an emission maximum at 460 and 480 nm, respectively, which blue-shifted from those measured in buffer and nearly identical to those measured in EtOH and EtOH:buffer (1:1) (Figure 1b,d). This indicates that the polarity of the probes environment is more hydrophobic than the buffer and these co-solvents can adequately represent the polarity of the probe environment in the cells.

In EtOH:MOPS buffer (1:1), SZnC showed a more than 8-fold increase in the fluorescence intensities upon addition of Zn^{2+} with minimal change of absorption spectra (Figure 2a,b). The dissociation constant (K_d) of SZnC calculated from the fluorescence titration curve was found to be 1.7 nM, which was nearly identical to that measured in the TP process (Figure 2c and Table 1). The Benesi-Hildebrand plots for Zn^{2+} binding showed a good linear relationship with slope 1.0, indicating formation of a 1:1 complex between the probe and Zn^{2+} (Figure S4, Supporting Information). This probe showed high selectivity for Zn^{2+} over 1 mM Na^+, K^+, Ca^{2+}, and Mg^{2+} and 1 μM Mn^{2+}, and modest selectivity over 1 μM Ni^{2+}, Pb^{2+}, Co^{2+}, and Cd^{2+} (Figure S2, Supporting Information). In the presence of 1 μM Fe^{2+} and Cu^{2+} the fluorescence was quenched, a result of metal-to-ligand electron transfer upon excitation, as previously reported.\(^{18}\) Further, the emission intensity of SZnC was pH-insensitive in the biologically relevant pH range (Figure S2, Supporting Information). The TP action cross section values (δΦ_{max}, where Φ is fluorescence quantum yield and δ is TP absorption cross section) of SZnC in the absence and presence of excess Zn^{2+} were determined to be 16 and 92 GM (Figure 2d and Table 1), while the Φ were 0.12 and 0.93, respectively. Similar results were obtained with SZn (Table 1 and Figure S3f, Supporting Information). The combined results established that these probes can serve as a TP turn-on probe to detect [Zn^{2+}] in the nanomolar range with minimal interference from other competing metal ions and pH values.

![Fig. 1](image1.png)  
**Fig. 1** TPM images of HeLa cells labeled with 2 μM (a) SZn and (c) SZnC. The images were merged with the corresponding DIC images. (b,d) Normalized emission spectra of (b) SZn, and (d) SZnC in 1,4-dioxane, EtOH, EtOH:Buffer (1:1), Buffer (30 mM MOPS, 10 mM EGTA, 100 mM KCl, pH 7.2), and HeLa cells (indicated in green). Excitation wavelength for TPM was 750 nm, and excitation wavelengths for one-photon spectra were 376 nm for SZn and 388 nm for SZnC. Scale bar = 48 μm. Cells shown are representative images from replicate experiments (n = 5).

![Fig. 2](image2.png)  
**Fig. 2** (a) Absorption spectra of SZnC in the absence (black line) and presence (red line) of excess Zn^{2+} (127 nM). (b) Fluorescence spectra of 1 μM SZnC in the presence of free Zn^{2+} (0 to 127 nM). The excitation wavelength was 388 nm. (c) One-photon (●) and two-photon (■) fluorescence titration curves for the complexation of SZnC with free Zn^{2+} (0 to 127 nM). (d) Two-photon action spectra of SZnC in the absence (●) and presence (■) of excess Zn^{2+} (127 nM). All data measured in 1:1 EtOH:MOPS buffer (30 mM MOPS, 100 mM KCl, EGTA 10 mM, pH = 7.2).

<table>
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<tr>
<th>Compd.</th>
<th>λ_{em}^{(1)} (nm)</th>
<th>λ_{em}^{(2)} (nm)</th>
<th>Φ^{(e)}</th>
<th>K_d (nM)</th>
<th>FEP</th>
<th>λ_{max}^{(2)} (nm)</th>
<th>ΔΦ^{(e)}</th>
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<td>750</td>
<td>9</td>
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<td>375</td>
<td>481</td>
<td>1.00</td>
<td>750</td>
<td>98</td>
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<td></td>
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<tr>
<td>SZnC</td>
<td>388</td>
<td>499</td>
<td>0.12</td>
<td>1.7 (1.5)</td>
<td>8.5</td>
<td>750</td>
<td>16</td>
</tr>
<tr>
<td>SZnC + Zn^{2+}</td>
<td>393</td>
<td>499</td>
<td>0.93</td>
<td>750</td>
<td>92</td>
<td></td>
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</tbody>
</table>

\(^{a}\) All data measured in 1:1 EtOH:MOPS buffer (30 mM MOPS, 100 mM KCl, EGTA 10 mM, pH = 7.2). \(^{b}\) F_{max} of the one-photon absorption in nm. \(^{c}\) ΔΦ_{max} of excess Zn^{2+} (127 nM).

Fluorescence quantum yield. The uncertainty is ± 10%. \(^{d}\) Dissociation constants for Zn^{2+}, the uncertainty is ± 10%. The number in parentheses is measured by two-photon process. \(^{e}\) Fluorescence enhancement factor, (F_{max}(Zn^{2+})/F_{max}(Zn^{2+}).

\(^{f}\) Two-photon action cross section in GM (1 GM = 10^{-50} cm^2 s photon^{-1}). The uncertainty is ± 15%.
To investigate the cellular location of SZnC, we conducted co-localization experiments with SZnC and BODIPY TR ceramide (BTC), a well-known red-emissive marker for Golgi apparatus. The TPM and confocal microscopy images of HeLa cells labeled with SZnC and BTC were obtained by using the detection windows 450-550 and 600-700 nm with equal intensities (Figure 3a-c). Both the images overlapped well with the Pearson’s co-localization coefficient value, A, equal to 0.92. Similar results were observed with A431, Raw 264-7, and HepG2 cells (Figure S5, Supporting Information). The co-localization experiments with SZnC and other markers for lysosome, mitochondria, and ER were also conducted (Figure 3d-i). The A value were founded to be 0.14, 0.21, and 0.31 with the markers for lysosome, mitochondria, and ER, respectively. These results clearly indicate that SZnC existed predominantly in the Golgi apparatus.

It has been theoretically predicted that for a small molecule probe to be retained within the Golgi apparatus, its lipophilicity value, termed the log P value, should fall within the range of 3-5. We determined the lipophilicity (log P oct) of the compounds by measuring its partitioning ratio between n-octanol and buffer (Table S1, Supporting Information). Interestingly, the log P oct value of SZnC is 2.9 ± 0.1, which is slightly higher than that for SZn (2.5 ± 0.1) and reasonably matched with the theoretical prediction. We then performed a control experiment using 1, in which the pyridyl moiety at MeO-DPEN in SZnC was replaced by benzyl unit. The log P oct value of 1 is 3.1 ± 0.1, a nearly identical value with that for SZnC. However, the TPM image of 1-labeled HeLa cells showed the intense TPEF throughout the entire cell excepting the nucleus as we observed with SZn (Figure S6, Supporting Information). Because it has been well established that the pH of Golgi apparatus is slightly acidic (6.0-6.7), the Golgi localization of the probe might be linked to the weak base such as pyridyl group. Similar results were reported. Hence, both the lipophilicity and the pyridyl moiety might be responsible for SZnC to reside in Golgi apparatus.

![Fig. 3](image)

**Fig. 3** (a,d,g,j) TPM, (b,e,h,k) OPM images of HeLa cells co-labeled with SZnC (2 μM) and organelle markers. (c,f,i,l) Merged images. The excitation wavelengths for TPM and OPM are 750 nm and 552 nm, respectively. Scale bars = (a,d) 7, (g) 8 and (j) 10 μm. Cells shown are representative images from replicate experiments (n = 10).

Next, we investigated whether SZnC could be used to monitor changes of [Zn2+]Golgi in live cells. First, SZnC showed negligible cytotoxicity as monitored by MTS assay (Figure S7, Supporting Information). The TPM imaging was performed at 37 °C using an incubating chamber to maintain the humidity and pH. Using excitation at 750 nm with femtosecond pulses, we collected the images at 2.0 sec intervals. Upon addition of N,N,N',N'-tetakis-(2-pyridylmethyl)-ethylenediamine (TPEN, a membrane permeable zinc chelator) to the imaging solution, the TPEF intensities were reduced (Figure 4a,b). The addition of ZnCl2 with ionophore pyrithione (2-mercaptopyridine-N-oxide) caused a prompt increase of TPEF more than 3-fold and the signal returned to a weak level upon treatment with TPEN. These outcomes established that SZnC is able to detect the changes of [Zn2+]Golgi in real-time. In addition, three dimensional (3D) multicolor image of live cells were constructed from 200 TPM sections with 0.1 μm intervals. The 3D image clearly showed the local distribution of Zn2+ ions in Golgi apparatus along with the location of the ER and nucleus (Figure 4c and Video S1, Supporting Information).

![Fig. 4](image)

**Fig. 4** (a) TPM images of 2 μM SZnC-labeled HeLa cells before and after addition of TPEN and ZnCl2/pyrithione to the imaging solution. Before (0 s) and after (225 s) addition of 250 μM TPEN, and then after (372 s) addition of 50 μM ZnCl2/pyrithione, and after (712 s) addition of 250 μM TPEN. (b) The relative TPEF intensity of SZnC-labeled HeLa cells as a function of time. (c) 3D reconstructed and three color TP image of the HeLa cells. The cells co-labeled with SZnC (2 μM), Hoechst 33342 (0.5 μM) for nucleus staining, and ER-Tracker Red (1 μM). 200 TPM images were collected along the z direction with 100 × magnification at depths of 0–20 μm from the bottom of the cells. The TPEF intensities were collected at 400–600 nm upon excitation at 750 nm with fs pulse. Scale bar in (a): 16 μm. Cells shown are representative images from replicate experiments (n = 10).

Finally, we conducted the usefulness of SZnC in monitoring [Zn2+]Golgi in a fresh rat hippocampal slice, a part of the brain that is primarily responsible for learning and memory. The TPM images of a fresh rat hippocampal slice incubated with 20 μM SZnC displayed strong fluorescence throughout the pyramidal neuron layers especially in the dentate gyrus (DG) and CA3 regions (Figure 5a). When tissue
slices were first treated with ZnCl₂ with pyrithione, the TPEF intensities in the DG region increased dramatically and decreased upon treatment of TPEN (Figure 5c-g) as we had observed in the live cell imaging. This result indicated that the [Zn²⁺]<sub>Golgi</sub> Moreover, the TPM images showed that [Zn²⁺]<sub>Golgi</sub> can be observed in the individual cells at a depth of more than 100 µm (Figure S8a,b, Supporting Information). In addition, SZNc displayed high photostability as tested by collecting TPEF for 1 h in the tissue (Figure S8c,d, Supporting Information). These results conclusively demonstrated the capability of SZNc to monitor [Zn²⁺]<sub>Golgi</sub> in live cells and living tissues through the use of TPM.

In summary, we have developed a Golgi-localizable TP fluorescents probe for detecting Zn²⁺ ions (SZnC). This probe is characterized by an 8-fold turn-on response in the presence of Zn²⁺ ions with maximum δΦ<sub>max</sub> values of 92 GM, with pH insensitivity in the physiologically relevant range, Golgi-localizing ability, low cytotoxicity, and high photostability, thereby allowing direct visualization of [Zn²⁺]<sub>Golgi</sub>. The TPM imaging revealed that this probe allows for real-time monitoring of the changes of [Zn²⁺]<sub>Golgi</sub> as well as their 3D distributions in live cells and tissues. These results demonstrate that this probe will find useful applications in understanding the Golgi-related biology as well as for pharmaceutical research.

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Notes and references

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Fig. 5 TPM images of a rat hippocampal slice stained with 20 µM SZNc for 1h. (a) 150 TPM images along the z-direction at the depth of approximately 100-200 µm were accumulated with magnification 10x. (b) Bright-field image. (c-f) Magnification at 20x in the dentate gyrus (DG) regions (white box in (a) at a depth of 120 µm. (c) Bright-field image, (d-f) TPM images before (d) and after (e) addition of 200 mM Zn²⁺/pyrithione to the imaging solution. (f) After addition of 1 mM TPEN to (e). (g) Relative TPEF intensity of (d-f) as a function of time. The TPEF intensities were collected at 400 – 600 nm upon excitation at 750 nm with fs pulse. Scale bars: (a) 300, and (f) 100 µm. Images shown are representative images from replicate experiments (n = 6).

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