Supporting Information

Detection of Mercury in Fish Organs with a Two-Photon Fluorescent Probe

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before imaging. *(right)* Relative TPEF intensity measured at A–D in the *left* panel as a function of time. The TPEF was collected at 500-620 nm upon excitation at 780 nm with fs pulse. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 μm.

**(S9)** Figure S5. (a) TPM image of AHg1-labeled HeLa cells treated with HgCl₂. The cells were incubated with AHg1 (2 μM) for 20 min at 37 °C and HgCl₂ (10 μM) was added immediately before imaging. (b) Image of HeLa cells in panel (a) after incubation with Hoechst 33342 (1 μM) for an additional 10 min at RT. The emission was collected at 500-620 nm (AHg1) (a), and 450-550 nm (Hoechst 33342) (b), respectively. (c) Co-localized images. (d) Bright field image. The wavelengths for excitation were 780 nm, respectively. Scale bar, 30 μm. Cells shown are representative images from replicate experiments (n = 5).

**(S10)** Preparation and staining of *Oryzias latipes* organs

**(S10)** Figure S6. TPM and bright field images of AHg1-labeled (10 μM) kidney, heart, gill and liver of *Oryzias latipes* by magnification at 10×. The TPEF was collected at 500-620 nm upon excitation at 780 nm with fs pulses. Scale bar, 300 μm.

**(S11)** Inductively coupled plasma mass spectrometry (ICP MS)
Synthesis of AHg1. Synthesis of AHg1 is summarized in Scheme 1. 6-Acyl-2-[N-methyl-N-(carboxymethyl)amino]naphthalene\(^1\) (Acadan) was available from our previous work. Synthesis of other compounds is described below.

2-Methoxy-\(N,N\)-bis(2-hydroxyethyl)aniline (A). A mixture of o-anisidine (10 g, 81 mmol) and ClCH\(_2\)CH\(_2\)OH (43 g, 535 mmol) in H\(_2\)O (200 mL) was stirred for 72 h at 110 °C. The resulting mixture was filtered, extracted with CH\(_2\)Cl\(_2\), dried over MgSO\(_4\), filtered, and evaporated in vacuo to obtain yellow oil. Yield 17.1 g (100 %); IR (KBr): 3459 cm\(^{-1}\); \(^1\)H NMR (400MHz, CDCl\(_3\)): \(\delta\) 7.05 (1H, dd, \(J=7.6, 1.6\) Hz), 6.96 (1H, ddd, \(J=7.6, 7.6, 1.6\) Hz), 6.75-6.81 (2H, m), 3.85 (2H, br s), 3.68 (3H, s), 3.41 (4H, t, \(J=5.4\) Hz), 3.08 (4H, t, \(J=5.4\) Hz); \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 155.1, 138.5, 125.3, 124.8, 121.4, 111.8, 60.0, 56.8, 55.5; Anal. Calcd for C\(_{11}\)H\(_{17}\)NO\(_3\): C, 62.54; H, 8.11; N, 6.63. Found: C, 62.42; H, 8.08; N, 6.66.

2-Methoxy-4-nitro-\(N,N\)-bis(2-hydroxyethyl)aniline (B). A mixture of A (2.0 g, 9.5 mmol), HOAc (32 g, 530 mmol) in H\(_2\)O (200 mL) was stirred for 10 min at RT. To this mixture, NaNO\(_3\) (0.72 g, 10 mmol) was added and stirred for 2 h at RT. The resulting mixture was filtered, extracted with CH\(_2\)Cl\(_2\), dried over MgSO\(_4\), filtered, and evaporated in vacuo to obtain yellow oil. Yield 2.3 g (94 %); IR (KBr): 3583, 1518 cm\(^{-1}\); \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.76 (1H, dd, \(J=9.0, 2.4\) Hz), 7.65-6.81 (2H, m), 3.85 (2H, br s), 3.68 (3H, s), 3.41 (4H, t, \(J=5.0\) Hz), 3.08 (4H, t, \(J=5.0\) Hz); \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 151.1, 138.5, 125.3, 124.8, 121.4, 111.8, 60.0, 56.8, 55.5; Anal. Calcd for C\(_{11}\)H\(_{16}\)N\(_2\)O\(_5\): C, 52.42; H, 8.11; N, 10.63. Found: C, 52.97; H, 5.82; N, 11.38.

2-Methoxy-4-nitro-\(N,N\)-bis(2-tosylethyl)aniline (C). A mixture of B (2.0 g, 7.8 mmol) and NaOH (aq) (1.1 M, 100 mL) was stirred at 0 °C and \(p\)-toluenesulfonyl chloride (3.7 g, 19 mmol) in THF (100 mL) was added slowly. The mixture was stirred for 1 h at 0 °C and warmed to RT. The resulting
mixture was filtered, extracted with CH$_2$Cl$_2$ and brine, dried over MgSO$_4$, filtered, and then concentrated in vacuo to obtain brown oil. Yield 3.4 g (78 %); IR (KBr): 1514, 1355 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.65-7.70 (5H, m), 7.58 (1H, d, $J = 2.4$ Hz), 7.27 (4H, d, $J = 8.7$ Hz), 6.63 (1H, d, $J = 8.7$ Hz), 4.06 (4H, t, $J = 5.8$ Hz), 3.81 (3H, s), 3.61 (4H, t, $J = 5.8$ Hz), 2.42 (6H, s); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 150.7, 145.1, 143.0, 141.5, 132.5, 129.8, 127.8, 117.6, 117.5, 107.2, 67.3, 55.9, 51.7, 21.6; Anal. Calcd for C$_{25}$H$_{28}$N$_2$O$_9$S$_2$: C, 53.18; H, 5.00; N, 4.96; S, 11.36. Found: C, 51.11; H, 4.77; N, 4.86; S, 11.40.

$N$-(2-Methoxy-4-nitrophenyl)-4,7,10,13-tetrathia-1-aza-15-crown-5 (D). A mixture of 3,6-dithiaoctan-1,8-dithiol (1.3, 5.8 mmol) and CsCO$_3$ (2.3 g, 6.9 mmol) in DMF (100mL) was slowly added to a solution of C (3.0 g, 5.31 mmol) in DMF (300 mL) at RT over the period of 3 h. The mixture was warmed to 60 °C and stirred for 48 h. The solvent was evaporated and the residue was transferred to H$_2$O (100 mL) and stirred for 1 hr. The product was extracted with CH$_2$Cl$_2$, dried over MgSO$_4$, filtered, and evaporated. The product was purified by flash column chromatography using n-hexane/ethyl acetate (3:1 to 1:1) as the eluent and concentrated under reduced pressure to obtain a brown powder. Yield 0.42 g (18 %); mp 147.6 °C; IR (KBr): 1520 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.83 (1H, dd, $J = 8.9, 2.4$ Hz), 7.70 (1H, d, $J = 2.7$ Hz), 6.77 (1H, d, $J = 8.9$ Hz), 3.93 (3H, s), 3.56 (4H, t, $J = 6.5$ Hz), 2.78-2.90 (16H, m); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 165.2, 149.5, 144.9, 118.4, 115.7, 107.6, 56.3, 33.2, 33.1, 33.0, 30.5; Anal. Calcd for C$_{17}$H$_{26}$N$_2$O$_3$S$_4$: C, 46.98; H, 6.03; N, 6.44; S, 29.51. Found: C, 47.02; H, 6.05; N, 6.21; S, 29.71.

$N$-(4-Amino-2-methoxyphenyl)-4,7,10,13-tetrathia-1-aza-15-crown-5 (E). A mixture of D (1.8 g, 4.5 mmol) and SnCl$_2$ (10 g, 44 mmol) in THF/EtOH (1/1, 150 mL) was refluxed for 12 hr. The solvent was removed in vacuo and the product was stirred in NaOH solution. After the solution became basic, the product was extracted with CH$_2$Cl$_2$, dried over MgSO$_4$, and the solvent was removed in vacuo to obtain dark brown oil. Yield 1.0 g (56 %); IR (KBr): 3340, 3472 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 6.84 (1H, d, $J = 8.0$ Hz), 6.25-6.20 (2H, m), 3.79 (3H, s), 3.24 (4H, t, $J = 7.5$ Hz), 2.84-2.77 (12H, m), 2.60 (4H, t, $J = 7.5$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 153.43, 142.94, 134.43, 120.37, 106.70, 99.86, 55.35, 53.92, 32.92, 32.65, 32.56, 30.90; Anal. Calcd for C$_{17}$H$_{28}$N$_2$O$_5$S$_4$: C, 50.46; H, 6.97; N, 6.92; S, 31.69. Found: C, 50.51; H, 6.85; N, 7.08; S, 32.02.

AHg1. A mixture of 6-acyl-2-[N-methyl-N-(carboxymethyl)amino]naphthalene$^{[1]}$ (0.05 g, 0.21 mmol), 1-hydroxybenzotriazole (0.03 g, 0.25 mmol), and N,N'-dicyclohexylcarbodiimide (0.05 g, 0.21 mmol) in CH$_2$Cl$_2$ (10 mL) was stirred for 1 hr. To this mixture, compound E (0.08 g, 0.21 mmol) in CH$_2$Cl$_2$ were added and stirred for 4 hr under N$_2$. The resulting mixture was filtered and the filtrate was extracted with saturated NaHCO$_3$ (aq), dried over Na$_2$SO$_4$, filtered and evaporated. The crude product was purified by column chromatography using Hexane/ethyl acetate/CHCl$_3$ (1:1:1) as the eluent. Yield 0.064 g (50 %); mp 144.3 °C; IR (KBr): 1672, 1612 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.35 (1H, d, $J = 2.4$ Hz), 8.16 (1H, br s), 7.98 (1H, dd, $J = 1.6, 8.8$ Hz), 7.89 (1H, d, $J = 9.2$ Hz), 7.71 (1H, d, $J = 8.4$ Hz), 7.34 (1H, d, $J = 2.4$ Hz), 7.17 (1H, dd, $J = 2.4, 9.2$ Hz), 7.07 (1H, d, $J = 2.4$ Hz), 6.87 (1H, d, $J = 8.4$ Hz), 6.80 (1H, d, $J = 8.4$ Hz), 4.11 (2H, s), 3.83 (3H, s), 3.29 (4H, t, $J = 7.0$ Hz), 3.20 (4H, t, $J = 7.0$ Hz).
3.24 (3H, s), 2.27-2.81 (12H, m), 2.62 (4H, t, J = 7.0 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 197.7, 167.9, 154.3, 149.0, 137.1, 134.7, 133.3, 132.1, 131.3, 130.1, 126.8, 126.5, 125.0, 122.6, 116.4, 111.7, 107.5, 104.7, 59.4, 55.6, 53.8, 40.2, 32.7, 32.6, 32.5, 30.3, 26.5; Anal. Calcd for C$_{32}$H$_{41}$N$_3$O$_3$S$_4$: C, 59.69; H, 6.42; N, 6.53; S, 19.92. Found: C, 57.72; H, 6.27; N, 6.48; S, 19.81.

**Spectroscopic measurements.** Absorption spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer, and fluorescence spectra were obtained with Amico-Bowman series 2 luminescence spectrometer with a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using Coumarin 307 as the reference by the literature method.$^{[2]}$

![Figure S1](image)

**Figure S1.** (a) Normalized absorption and (b) emission spectra of AHg1 in 1,4-dioxane, DMF, EtOH, and H$_2$O.

**Table S1.** Photophysical properties of AHg1 in various solvents.

<table>
<thead>
<tr>
<th>Solvent ($E_T^{N}$)$^{[a]}$</th>
<th>$\lambda_{\text{max}}^{(1)}$ $^{[b]}$</th>
<th>$\lambda_{\text{fl}}^{\text{max}}$ $^{[b]}$</th>
<th>$\Phi$ $^{[c]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-dioxane (0.164)</td>
<td>329</td>
<td>426</td>
<td>0.062</td>
</tr>
<tr>
<td>DMF (0.386)</td>
<td>374</td>
<td>448</td>
<td>0.066</td>
</tr>
<tr>
<td>EtOH (0.654)</td>
<td>375</td>
<td>475</td>
<td>0.037</td>
</tr>
<tr>
<td>H$_2$O (1.000)</td>
<td>361</td>
<td>489</td>
<td>0.025</td>
</tr>
</tbody>
</table>

$^{[a]}$ The numbers in the parenthesis are normalized empirical parameter of solvent polarity.$^{[3]}$ $^{[b]}$ $\lambda_{\text{max}}$ of the one-photon absorption and emission spectra in nm. $^{[c]}$ Fluorescence quantum yield. The uncertainty is ± 15%.
**Determination of Apparent Dissociation Constants.** A series of solutions containing various [Hg\(^{2+}\)] was prepared in the presence of 1 μM AHg1 in 20 mM HEPES buffer and they were adjusted to 7.0 by the addition of 0.4 mM KOH.

The apparent dissociation constant \((K_d)\) was determined using the following equation: \(F - F_{\text{min}} = [\text{Hg}^{2+}] (F_{\text{max}} - F_{\text{min}})/(K_d + [\text{Hg}^{2+}])\), where \(F\) is the observed fluorescence, \(F_{\text{max}}\) is the fluorescence for the Hg\(^{2+}\)-AHg1 complex, and \(F_{\text{min}}\) is the fluorescence for the free AHg1. The \(K_d\) value that best fits the titration curve (Figures 1b, S3 and S4) with the equation was calculated by using the Excel program as reported.[4]

In order to determine the \(K_d^{\text{TP}}\) for the two-photon process, the TPEF intensity were recorded in the range of 500-620 nm with a DM IRE2 Microscope (Leica) excited by a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane.

**Figure S2.** (a) Two-photon emission spectra of AHg1 in the presence of free Hg\(^{2+}\) (0–2.35 μM). (b) Hill plots for the complexation of AHg1 with free Hg\(^{2+}\) (0–2.35 μM). These data were measured in 20 mM HEPES buffer (pH 7.0). The excitation wavelengths for one- and two-photon processes were 365 and 780 nm, respectively.
Measurement of Two-Photon Cross Section. The two-photon cross section ($\delta$) was determined by using femto second (fs) fluorescence measurement technique as described.[5] AHg1 was dissolved in 20 mM HEPES buffer (pH 7.0) at concentrations of $5.0 \times 10^{-6}$ M and then the two-photon induced fluorescence intensity was measured at 740–940 nm by using fluorescein ($8.0 \times 10^{-5}$ M, pH = 11) as the reference, whose two-photon property has been well characterized in the literature.[6] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_r \Phi_r c_r)/(S_s \Phi_s c_s)$, where the subscripts $s$ and $r$ stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as $S$. $\Phi$ is the fluorescence quantum yield. $\phi$ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as $c$. $\delta_r$ is the TPA cross section of the reference molecule.

Cell Culture and Imaging. HeLa human cervical carcinoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM (WelGene Inc, Seoul, Korea) supplemented with 10% FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 ug/ml). All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of CO$_2$/air at 37 °C. Two days before imaging, the cells were passed and plated on glass-bottomed dishes (MatTek). For labeling, the growth medium was removed and replaced with DMEM without FBS. The cells were incubated with 2 µM AHg1 for 20 min at 37 °C and were washed three times with DMEM without FBS. Then 20 µM HgCl$_2$ was added to the cells and imaged. To confirm whether the TPEF was due to the AHg1-Hg$^{2+}$ complex, TPEN (1 mM) was added to the HgCl$_2$ treated cells and imaged after 5 min.

Two-Photon Fluorescence Microscopy. Two-photon fluorescence microscopy images of AHg1-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a ×100 (NA = 1.30 OIL) and ×10 (NA = 0.30 DRY) objective lens, respectively. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 360–460 nm and 500–620 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512 × 512 pixels at 400Hz scan speed.

The pseudocolored TPM images of cultured HeLa cells treated with 2 µM AHg1 showed intense spots and homogeneous domains with two-photon emission maxima at 436 (blue) and 484 nm (red), respectively (Figures S3). The TPEF spectrum of the intense spots was asymmetrical and could be fitted to two Gaussian functions with emission maxima at 430 (green) and 475 nm (orange), whereas the TPEF spectrum of the homogeneous domain could be fitted to a single Gaussian function (pink) with an emission maximum at 483 nm. It is to be noted that the longer wavelength band of the
dissected Gaussian function (orange) is similar to the band of the single Gaussian function (pink). This result suggests that the probe is located in two regions of different polarity: a more polar one that is likely to be cytosol and a less polar one that is likely to be membrane-associated. Moreover, the shorter wavelength band (green) in the dissected Gaussian functions decreases to the baseline at wavelength of < 500 nm. Consistently, the TPM image collected at 500–620 nm is homogeneous without intense spots (Figure S3D), whereas the one collected at 360–460 nm clearly shows them (Figure S3C). Therefore, cytosolic AHg1 can be selectively detected by using the detection window of 500–620 nm, with minimum interference from the membrane-bound probes.

**Figure S3.** Pseudo colored TPM images of AHg1-labeled HeLa cells treated with HgCl₂. The cells were incubated with AHg1 (2 μM) for 20 min at 37 °C and Hg²⁺ (10 μM) was added immediately before imaging. TPEF was collected at 360–620 nm (A), 360–460 nm (C), and 500–620 nm (D), respectively. (B) Two-photon excited fluorescence spectra from the hydrophobic (blue) and hydrophilic (red) regions of AHg1-labeled HeLa cells treated with HgCl₂. The sky blue and pink curves represent the dissected Gaussian functions for the blue and red bands, respectively. The excitation wavelength was 780 nm. The images shown are representative of the images obtained in the replicate experiments (n =5). Scale bar, 30 μm.
Figure S4. (left) TPM image of AHg1-labeled HeLa cells treated with Hg$^{2+}$. The cells were incubated with AHg1 (2 μM) for 20 min at 37 °C and Hg$^{2+}$ (10 μM) was added immediately before imaging. (right) Relative TPEF intensity measured at A–D in the left panel as a function of time. The TPEF was collected at 500-620 nm upon excitation at 780 nm with fs pulse. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 μm.

Figure S5. (a) TPM image of AHg1-labeled HeLa cells treated with Hg$^{2+}$. The cells were incubated with AHg1 (2 μM) for 20 min at 37 °C and Hg$^{2+}$ (10 μM) was added immediately before imaging. (b) Image of HeLa cells in panel (a) after incubation with Hoechst 33342 (1 μM) for an additional 10 min at RT. The emission was collected at 500-620 nm (AHg1) (a), and 450-550 nm (Hoechst 33342) (b), respectively. (c) Co-localized images. (d) Bright field image. The wavelengths for excitation were 780 nm, respectively. Scale bar, 30 μm. Cells shown are representative images from replicate experiments (n =5).
Preparation and staining of *Oryzias latipes* organs. *Oryzias latipes* were acquired from Korea National Institute of Environmental Research. For this experiment, 100 *Oryzias latipes*, approximately 5 month post-hatched and fully matured with an average body weight of 0.28 g and ~3.0 cm long, were acclimated in water tanks for one week. Growth conditions followed guidelines recommended by the international toxicity test protocol (OECD 1992; http://www.env.go.jp/chemi/kagaku/). The fishes were devided into two groups, half of them in aquaria containing 2 ppb of Hg\(^{2+}\) and the other half in aquaria without Hg\(^{2+}\), and reared for 1 and 3 days, according to the OECD guideline for testing of chemicals in a fish.\[^8\] The fishes were euthanized after stun by ice-cold artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM NaHCO\(_3\), 1.25 mM NaH\(_2\)PO\(_4\), 10 mM D-glucose, 2.4 mM CaCl\(_2\), and 1.3 mM MgSO\(_4\)), and kidney, gill, liver and heart were dissected. The organs were incubated with 10 \(\mu\)M of AHg1 in ACSF bubbled with 95% O\(_2\) and 5% CO\(_2\) for 30 min at 37 °C, then washed three times with ACSF, and transferred to glass-bottomed dishes (MatTek) for imaging by a spectral confocal multiphoton microscope.

![Figure S6](image)

**Figure S6.** TPM and bright field images of AHg1-labeled (10 \(\mu\)M) kidney, heart, gill and liver of *Oryzias latipes* by magnification at 10×. The TPEF was collected at 500-620 nm upon excitation at 780 nm with fs pulses. Scale bar, 300 \(\mu\)m.
Inductively Coupled Plasma Mass Spectrometry (ICP MS). ICP MS analysis of kidney sample was conducted in Korea Basic Science Institute. Briefly, OmniTrace Ultra grade nitric acid (EM Science) was used for digestion experiments. All Teflonware was rinsed with dilute nitric acid and millipore water before use. Microwave digestions were carried out using a CEM Discover Labmate microwave synthesizer. Samples of fish kidney (~2.5 mg) were digested in nitric acid (3 mL) at 180 °C with 300-watt microwave irradiation for 20 min. The samples were preserved in high-density polyethylene (HDPE) vials and flushed with nitrogen. The resulting solutions were neutralized with 10.0 M NaOH solution, diluted to 100 mL with distilled water, and the subjected to ICP MS in solution form.

References