Supplementary Information

Peptide-based Targeting of Fluorescent Zinc Sensors to the Plasma Membrane of Live Cells

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Page Contents

List of Supporting Figures and Tables

Materials and Methods

HPLC grade acetonitrile, anhydrous *N*,*N*-dimethylformamide (DMF), dichloromethane, 4-methylpiperidine, *N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triisopropylsilane, and palmitic acid were purchased from Sigma-Aldrich. FMOC-Pro-OH, FMOC-Asp(OtBu)-OH, and Rink amide AM resin (0.61 mmol/g, 100-200 mesh) were obtained from Novabiochem. FMOC-Lys(MTT)-OH was purchased from Aapptec. 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was procured from Oakwood Chemicals. *N*-(6-Methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ) was acquired from Enzo Life Sciences. Disposable 2.5-mL reaction vessels were ordered from Torviq. All solvents were reagent grade unless otherwise specified, and commercially available reagents were used as received. 6-CO_2H ZP1[1] and zinquin [2] were prepared according to literature procedures. Reverse-phase HPLC purifications were carried out on an Agilent Technologies 1200 Series HPLC system. Mass spectra were collected on an 1100-series Agilent LC/MSD ion trap. Aqueous solutions were prepared using Millipore water. Molecular biology grade piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) and 99.999% KCl were purchased from Aldrich. In order to remove adventitious metal ions, buffered solutions were treated with Chelex resin (Bio-Rad) according to manufacturer specifications. A 50 mM zinc(II) stock solution was prepared using 99.999% $ZnCl_2$ (Aldrich). UV-vis spectra were recorded on a Varian Cary 50 Bio UV-visible spectrophotometer. Fluorescence spectra were recorded on a Quanta Master 4 L-format scanning spectrofluorimeter (Photon Technology International). The acquisition temperature was kept at 25 ± 0.1 °C by circulating water bath. Sample solutions were placed in quartz cuvettes (Starna) with 1 cm path lengths. Stock solutions of Palm-ZP1 and Palm-ZQ were prepared in DMSO, partitioned in 50 µL aliquots and stored in the dark at – 40° C.

Peptide Synthesis

Peptides were manually synthesized according to a modified literature protocol[3] as outlined below.

Palm-ZP1 (Palmitic acid-PPPDDK(ZP1)-$CONH_2$)

Palm-ZP1 was synthesized on the 15-µmol scale using Rink amide AM resin. The resin was placed in a fritted 2.5-mL Torviq disposable syringe and swelled with 2 mL of anhydrous DMF for 1 hour prior to synthesis. *N*-terminal FMOC groups were removed by treating the resin with a solution of 20% 4-methylpiperidine in DMF (v/v) for a period of 10 min, followed by a 5 × 1.5 mL wash with DMF. For all coupling reactions, 60 µmol (4 equiv.) of FMOC-protected amino acids, palmitic acid, or 6-CO_2H ZP1 were combined as solids with 60 µmol (23 mg) of HATU. Immediately prior to coupling, solids were dissolved in 1.5 mL of a freshly prepared 10% DIPEA/DMF (v/v) solution, placed in the reaction vessel and shaken for 25 min. After the allotted time, the resulting solution was expelled from the syringe and the resin was washed with 5 × 1.5 mL of DMF. Following addition of all amino acids and the *N*-terminal palmitic acid moiety, the 4-methyltrityl group was removed from the *C*-terminal Lys according to manufacturer specifications.[4] Briefly, the resin was first exchanged into dichloromethane (DCM). A 3% TFA/DCM (v/v) solution was prepared, and the resin was mixed with the TFA/DCM mixture 2 × 1.5 mL for 10 min/ea. During equilibration, the TFA/DCM solution turned bright yellow, indicating that MTT was being liberated. After MTT deprotection, the resin was washed with 5 × 1.5 mL DCM followed by 5 × 1.5 mL DMF. The 6-CO_2H ZP1 was then coupled to the ε-amino group of the *C*-terminal Lys as described above. Following Palm-ZP1 synthesis, the resin was washed with 5 × 1.5 mL DCM and dried in vacuo for a period of ≥20 min prior to cleavage. Palm-ZP1 was cleaved from the resin by treating with a TFA/water/triisopropylsilane 95/2.5/2.5% (v/v) solution for 90 min. The resulting crude peptide was purified by HPLC on the semi-preparative scale using a C18 reverse-phase

column (VYDAC, 9.5 mm × 250 mm). A two-solvent system (A = 0.1% (v/v) TFA in H_2O; B = 0.1% TFA in acetonitrile (v/v)) was employed for purification according to the following protocol: isocratic flow, 20 % B, 0-5 min; gradient #1, 10-50% B, 5-10 min; gradient #2, 50-95% B, 10-35 min. The flow-rate was kept constant at 3 mL min^{-1} throughout the purification. Fractions from sequential runs containing Palm-ZP1 were pooled and lyophilized. The purity of the final product was assessed via analytical HPLC (Vydac, C18, 5 µm, 4.6 mm i.d. x 250 mm). After a 5 min isocratic wash, a linear gradient of 10-75% B was run over 30 min (35 min total) at a flow rate of 1 mL min^{-1}. Palm-ZP1 (retention time = 31.2 min) was judged to be ≥ 95% pure at all wavelengths based on the integrated chromatograms (**Figure S1**). Observed peaks for Palm-ZP1, $C_{92}H_{112}Cl_2N_{14}O_{17}$, in ESI-MS (m/z, amu; (calculated)): 1755.8 (1755.3) $[M+H]^+$, 878.2 (878.3) $[M+2H]^{2+}$, 585.7 (585.8) $[M+3H]^{3+}$ (**Figure S2**).

Palm-ZQ (Palmitic acid-PPPDDK(ZQ)-$CONH_2$)

Palm-ZQ was synthesized on the 15-µmol scale in a procedure similar to that used for Palm-ZP1. For conjugation of zinquin to the ε-amino group of the *C*-terminal Lys, 60 µmol (23 mg) of zinquin acid was mixed with 60 µmol (23 mg) of HATU in freshly prepared 10% DIPEA/DMF (v/v). The mixture was allowed to react with the resin for a period of 60 min, after which the resin was washed, cleaved, and purified. Palm-ZQ was purified via HPLC, using a two buffer system (*vide supra*), employing the following protocol: 1) isocratic flow, 10 % B, 0-2 min; 2) linear gradient A, 10-50 % B, 2-5 min; 3) linear gradient B, 50-99 % B, 5-25 min, all at a flow rate of 3 mL min^{-1}. Fractions containing Palm-ZQ from sequential runs were pooled and lyophilized. The purity of the final product was assessed via analytical HPLC (Vydac, C18, 5 µm, 4.6 mm i.d. x 250 mm). After an initial 5 min isocratic wash (10% B), a linear gradient of 10-95% B was run over 35 min (40 min total) at a flow rate of 1 mL min^{-1}. Palm-ZQ (retention time = 38.6 min) was judged to be ≥ 90% pure at all wavelengths based on the integrated chromatogram (**Figure S3**). Observed peaks for Palm-ZQ, $C_{64}H_{92}N_{10}O_{15}S$, in ESI-MS (m/z, amu; (calculated)): 1295.7 (1295.6) $[M+Na]^+$, 1274.0 (1273.6) $[M+H]^+$ (**Figure S4)**.

Photophyscial and Zinc-Binding Properties of Palm-ZP1

Spectroscopic measurements for Palm-ZP1 were carried out in a mixed solvent system consisting of 25 mM PIPES buffer (pH 7) with 50 mM KCl and 50% acetonitrile (v/v) (**Figure 2** and **S5**). Except where noted, all fluorescence data were obtained by exciting at 495 nm and observing from 500-650 nm, with 0.1 sec integration time and slit widths of 0.4 mm (1.6 nm). Emission spectra represent the average of three scans. The quantum yield of Palm-ZP1 was referenced to fluorescein in 0.1 M $NaOH_{(aq)}$, which has a known quantum yield of $\phi = 0.95$.[5]

Apparent $K_{d\text{-}Zn}$ for Palm-ZP1:

Apparent zinc-binding affinities ($K_{d\text{-}Zn}$) were determined by a modified literature procedure.[6] For each zinc-binding titration, 1 mM EDTA and 2 mM $CaCl_2$ were added to the MeCN/PIPES buffered solution. Palm-ZP1 or ZP1 was added, and the system was allowed to reach equilibrium (30 min). Aliquots of $ZnCl_2$ were successively added, and the emission spectra recorded once the emission spectrum stabilized (~30 min). The amount of free zinc for a given concentration of total zinc was calculated using the maxchelator program (http://maxchelator.stanford.edu/webmaxc/webmaxcS.htm) based on initial values of 1 mM EDTA, 2mM $CaCl_2$, an ionic strength of $I = 0.05$ mol dm^{-3} , and a pH of 7.0. Due to the addition of acetonitrile to the buffered solution, we compared the apparent $K_{d\text{-}Zn}$ for Palm-ZP1 to ZP1 under identical conditions (**Figure S5** and **Table S1**).

Photophysical and Zinc-Binding Properties of Palm-ZQ

Characterization of Palm-ZQ (**Figure 7**) was performed in 25 mM PIPES buffer (pH 7) with 50 mM KCl, and 50% (v/v) acetonitrile. Zinc affinities ($K_{d\text{-}Zn}$) were compared to TSQ under identical conditions (**Figure S9** and **Table S2**). Quantum yields for Palm-ZQ and TSQ were referenced to quinine sulfate in 0.1 M H_2SO_4, which has a known quantum yield of $\phi = 0.55$.[7]

Apparent $K_{d\text{-}Zn}$ for Palm-ZQ:

Apparent zinc-binding affinities ($K_{d\text{-}Zn}$) were determined using a modified literature procedure.[2] For each zinc-binding titration, 2 mM EGTA was added to the MeCN/PIPES buffered solution. Palm-ZQ or TSQ was added and the system was allowed to reach equilibrium before beginning the titration (30 min). Aliquots of $ZnCl_2$ were successively added, and the emission spectra recorded once the emission spectrum stabilized (~30 min). The amount of free zinc for a given concentration of total zinc was calculated using the maxchelator program (http://maxchelator.stanford.edu/webmaxc/webmaxcS.htm) based on initial values of 2 mM EGTA at an ionic strength of $I = 0.05$ mol dm^{-3} and a pH of 7.0. Due to the addition of acetonitrile to the buffered solution, we compared the apparent $K_{d\text{-}Zn}$ for Palm-ZQ to TSQ under identical conditions (**Figure S9** and **Table S2**).

Mammalian Cell Culture, Labeling, and Imaging Procedures.

General

HeLa cells were cultured at 37 °C under a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle Medium (High Glucose DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin (100 μg/mL), and streptomycin (100 μg/mL). RWPE-1 cells were cultured at 37 °C under a 5% CO_2 humidified atmosphere in keratinocyte serum-free media (Life Technologies) supplemented with prequalified human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE). For live cell imaging, cells were seeded in 35-mm poly- D-Lys coated glass-bottom culture dishes (MatTek Corporation).

Palm-ZP1

Palm-ZP1 (2.5 μM) was incubated for 30 min at 37° C and 5% CO_2 in dye- and serum-free DMEM. For multichannel imaging and colocalization studies, staining of the plasma membrane was accomplished by addition of Cell Mask Orange (Life Technologies, final concentration 2.5 μg/mL) for 15-20 min. Prior to imaging, cells were rinsed with warm dye- and serum-free DMEM (2 × 2 mL) and bathed in warm dye- and serum-free DMEM (2 mL). To assess the zinc responsiveness of Palm-ZP1, stock solutions of $ZnCl_2$ (10 mM) were diluted in warm dye- and serum-free DMEM to a final $ZnCl_2$ concentration of 50 μM. The zinc-enriched media was exchanged in the cell culture dish directly on the microscope stage. Similarly, a stock solution of ethylenediaminetetraacetic acid (EDTA, 100 mM) in water (pH 7) was diluted in warm dye- and serum-free DMEM to a concentration of 100 μM. Media containing EDTA was exchanged in the culture dishes on the microscope stage.

Palm-ZQ

Palm-ZQ (10 μM) was incubated for 30 min at 37° C and 5% CO_2 in dye- and serum free DMEM. For multi-channel imaging and colocalization studies, staining of the mitochondria was accomplished by addition of Mitotracker Red (Life Technologies, final concentration: 1 μM) for 30 min. Prior to imaging, cells were rinsed with warm dye- and serum-free DMEM (2 × 2 mL) and bathed in warm dye- and serum-free DMEM (2 mL). To assess the zinc responsiveness of Palm-ZQ, stock solutions of $ZnCl_2$ (10 mM) were diluted in warm dye- and serum-free DMEM to a final $ZnCl_2$ concentration of 50 μM. The zinc-enriched media was exchanged in the cell culture dish directly on the microscope stage. Similarly, a stock solution of ethylenediaminetetraacetic acid (EDTA, 100 mM) in water (pH 7) was diluted

in warm dye- and serum-free DMEM to a concentration of 100 μM. Media containing EDTA was exchanged in the culture dishes on the microscope stage.

Fluorescence Microscopy

The imaging experiments were performed using a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with an EM-CCD digital camera (Hamamatsu) and a MS200 XY Piezo Z stage (Applied Scientific Instruments). The light source was an X-Cite 120 metal-halide lamp (EXFO) and the fluorescence images were obtained using an oil-immersion objective at 63× magnification. The microscope was operated using Volocity software (Perkin-Elmer).

Quantification of Zinc Turn-On

Images were processed using ImageJ. All settings (i.e. exposure time and sensitivity) were kept constant for each image series.

Pearson's correlation coefficients (*r*):

To calculate *r*, images were first deconvoluted using a calculated point-spread function (PSF) map based on emission wavelength, refractive index of the media (n = 1.518), and the numerical aperture (1.4). Deconvoluted channels (i.e. sensor and organelle trackers) were merged and analyzed. For each image, a minimum of three regions-of-interest (ROI) were selected and the *r*-value calculated using an ImageJ plugin.[8] This process was repeated for 3-5 plates, which spanned multiple passage numbers (≤ 15) and days.

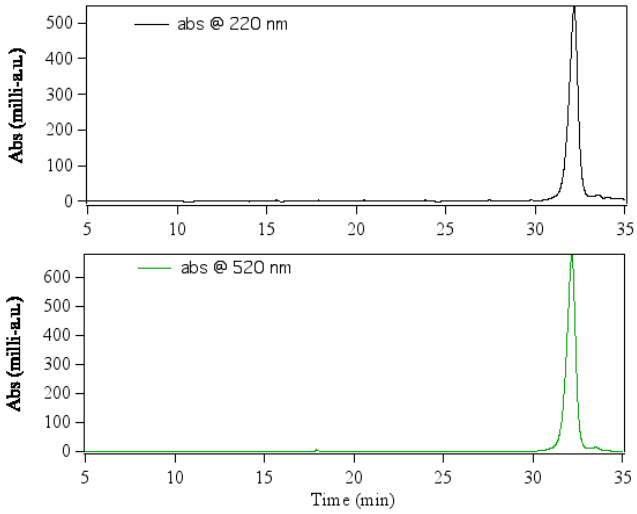

Figure S1. Analytical HPLC chromatogram of Palm-ZP1, monitoring absorbance at 220 and 520 nm. Palm-ZP1 was judged to be ≥ 95% pure based on the integrated absorbance signal at both wavelengths.

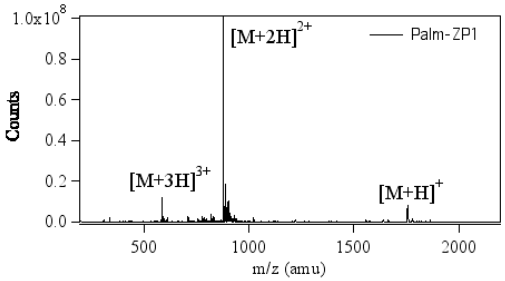

Figure S2. ESI-MS (positive mode) of Palm-ZP1.

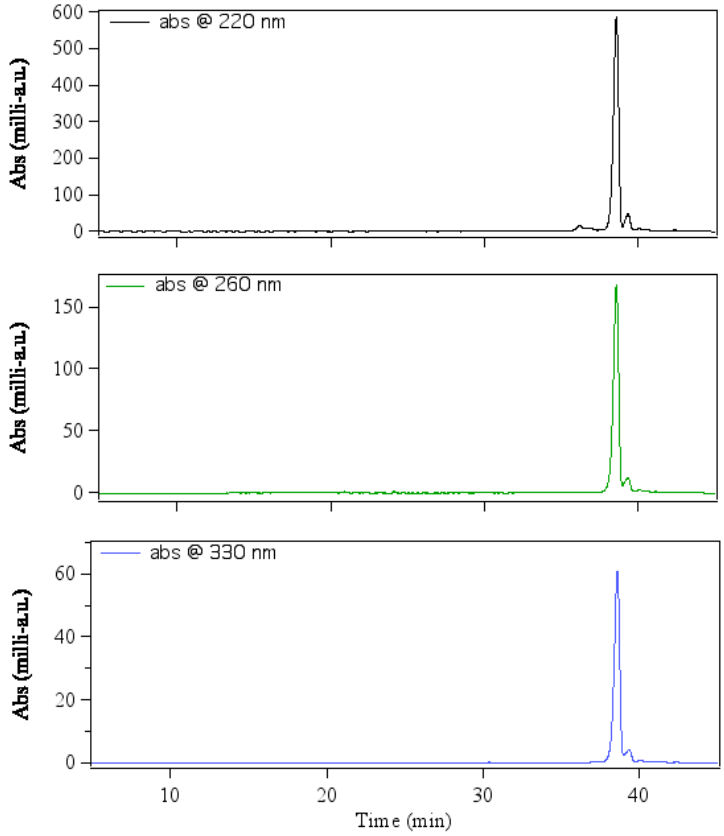

Figure S3. Analytical HPLC chromatogram of Palm-ZQ, monitoring absorbance at 220, 260, and 330 nm. Palm-ZQ was judged to be ≥ 90% pure based on the integrated absorbance signal of each wavelength.

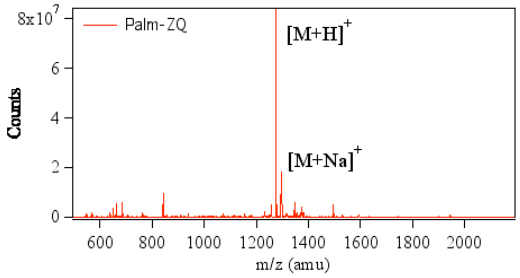

Figure S4. ESI-MS (positive mode) of Palm-ZQ.

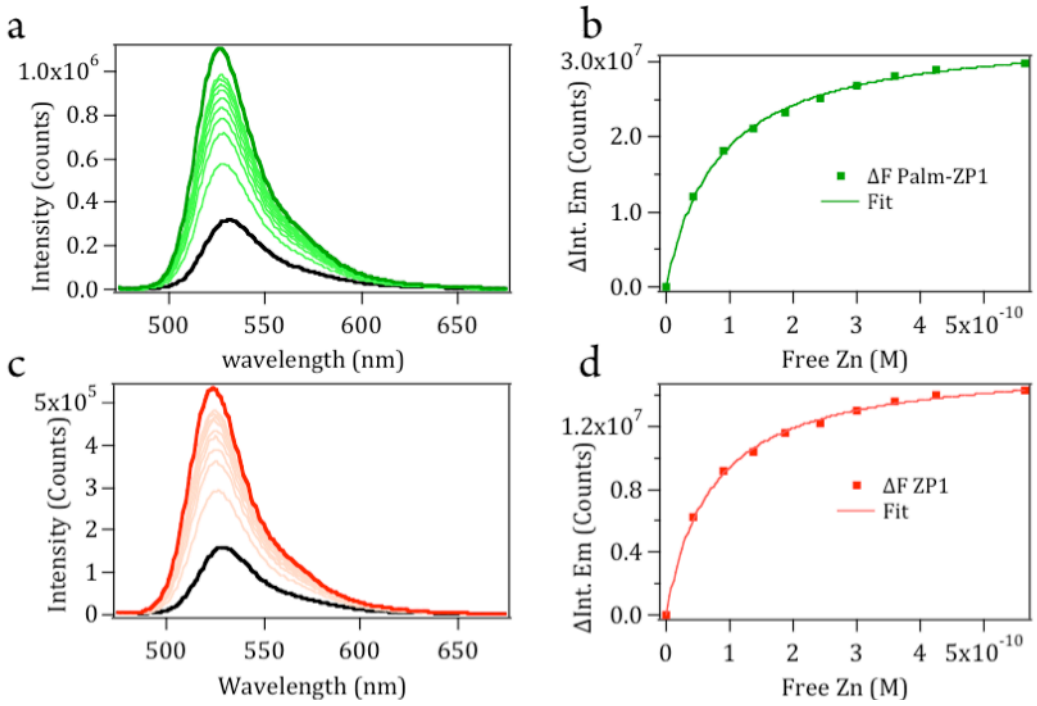

Figure S5. Measurement of the zinc-binding affinities, by fluorescence spectroscopy, for Palm-ZP1 (green) and ZP1 (red), in 25 mM PIPES buffer (pH 7) with 50 mM KCl, and 50% (v/v) acetonitrile. Changes in the emission spectra of Palm-ZP1 (a) and ZP1 (c) upon addition of increasing amounts of free zinc. Representative binding isotherms and fits for Palm-ZP1 (b) and ZP1 (d). Apparent $K_{d\text{-}Zn}$ values reported are the average of three trials and are listed in Table S1. Samples were excited at 470 nm with an emission window of 475-675 nm.

Table S1. Photophysical and zinc-binding properties of Palm-ZP1 and ZP1.

Sensor	Abs λ (nm), ε × 10^4 ($M^{-1}cm^{-1}$)		ϕ_{apo}	ϕ_{Zn}	$K_{d\text{-}Zn}$ (M)	Normalized $K_{d\text{-}Zn}$
	Apo	Zn(II)				
Palm-P_3D_2K(ZP1)[a]	517, 6.6	506, 7.4	0.16±0.053	0.79±0.049	8.2(3) × 10^{-11}	1.15
ZP1[b]	515, 7.9	507, 8.4	0.38	0.87	7.1(3) × 10^{-11} [c]	1

[a] Spectroscopic data and apparent zinc dissociation constants ($K_{d\text{-}Zn}$) were determined in 25 mM PIPES buffer (pH 7) with 50 mM KCl and 50% (v/v) acetonitrile. [b]Quantum yield, absorption, and extinction coefficient values were taken from literature.[1] [c]For comparison, the $K_{d\text{-}Zn}$ for ZP1 was determined under identical conditions. ZP1 has a known $K_{d\text{-}Zn}$ of 0.7 nM, in 50 mM PIPES buffer (pH 7), 100 mM KCl.

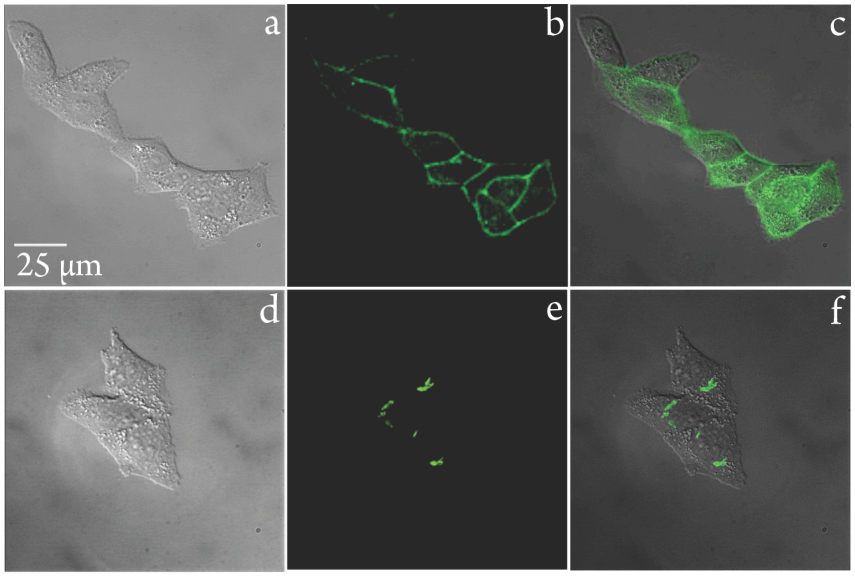

Figure S6. The observed localization of zinc-bound Palm-ZP1 and ZP1 in live HeLa cells. Top row: (a) DIC image of cells pretreated with a 2.5 μM solution of Palm-ZP1, (b) emission from zinc-bound Palm-ZP1, (c) overlay of (a) and (b). Bottom row: (d) DIC image of cells pretreated with 5 μM of ZP1 (e) emission from zinc-bound ZP1, (f) overlay of (d) and (e).

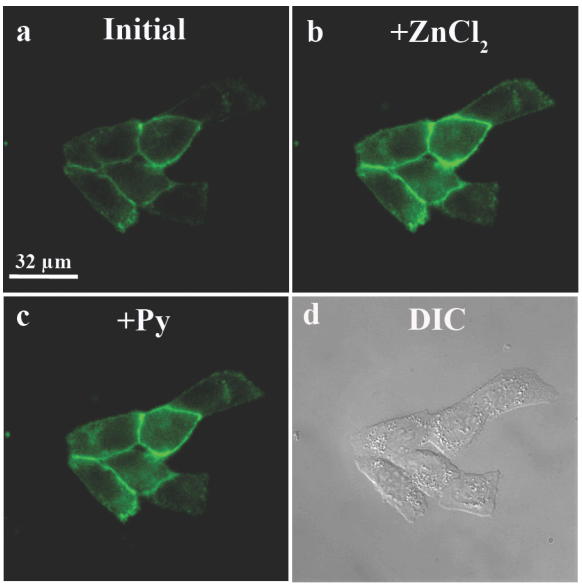

Figure S7. Imaging Palm-ZP1 in live HeLa cells via fluorescence microscopy. The fluorescence signal from Palm-ZP1, (a) initially, (b) after addition of 50 μM $ZnCl_2$, and (c) after addition of 100 μM sodium pyrithione, and (d) the DIC image for the set.

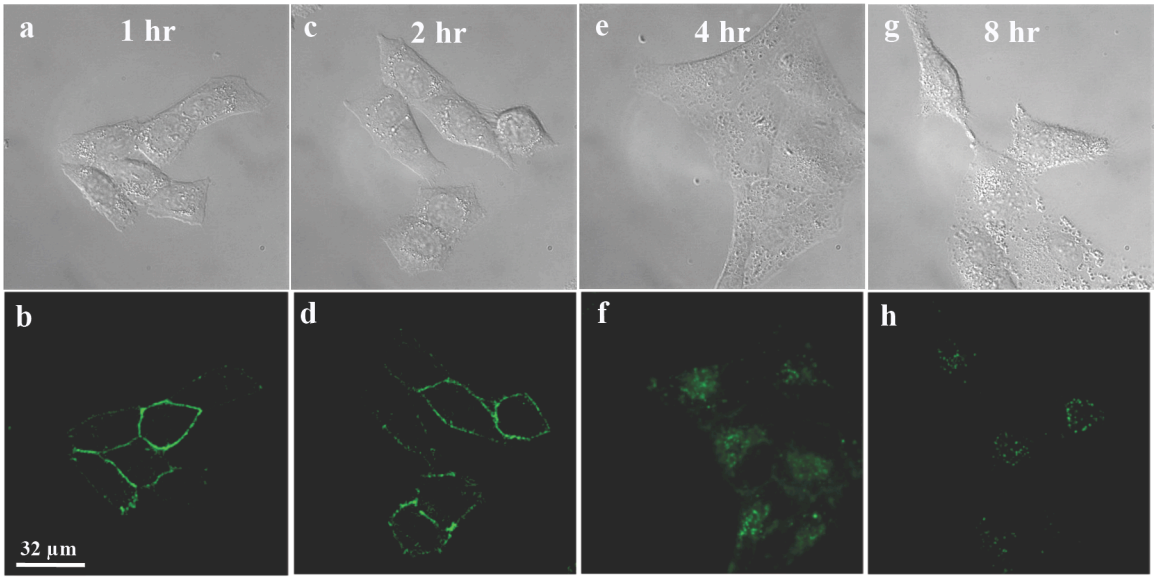

Figure S8. DIC and fluorescence microscopy images of HeLa cells that were incubated with Palm-ZP1 for 1 (a,b) 2 (c,d), 4 (e,f) or 8 hr (g,h) at 37 °C and 5 % CO_2.

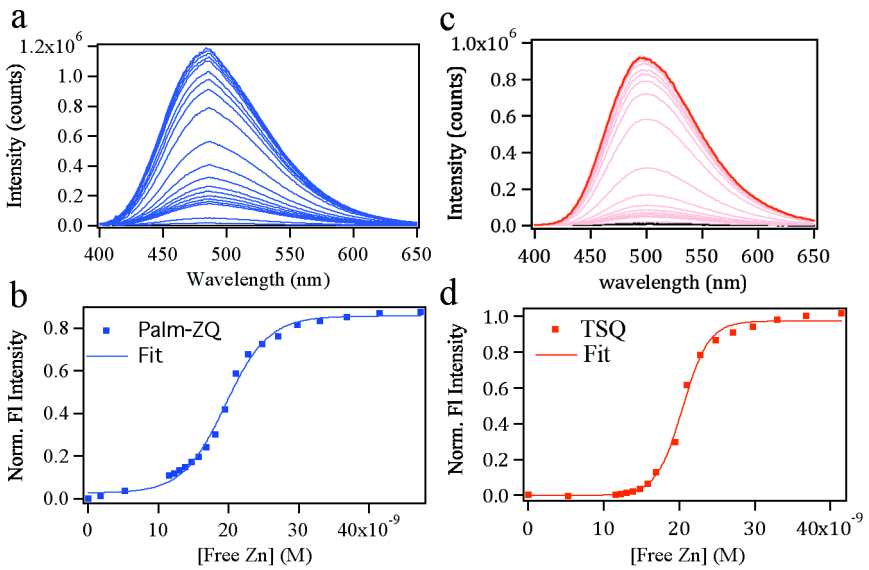

Figure S9. Measurement of the zinc-binding affinities, by fluorescence spectroscopy, of Palm-ZQ (blue) and TSQ (red) in 25 mM PIPES buffer (pH 7) with 50 mM KCl, and 50% (v/v) acetonitrile. Observed changes in the emission spectra of Palm-ZQ (a) and TSQ (c) upon addition of increasing amounts of free zinc. Representative normalized binding isotherm and fits for Palm-ZQ (b) and TSQ (d). Apparent $K_{d\text{-}Zn}$ values, which are the average of three trials, are given in Table S2.

Table S2. Photophysical and zinc-binding properties of Palm-ZQ and TSQ.

Sensor	Abs λ (nm), ε × 10^3 ($M^{-1}cm^{-1}$)		ϕ_{apo}	ϕ_{Zn}	$K_{d\text{-}Zn}$ (M)	Normalized $K_{d\text{-}Zn}$
	Apo	Zn(II)				
Palm-ZQ	244, 39.2 336, 3.9	263, 33.9 360, 3.6	≤ 0.014	0.35±0.02	1.95 (2) × 10^{-8}	0.975
TSQ	336, 3.5	360, 3.5	≤ 0.002	0.34±0.03	2.00 (2) × 10^{-8}	1

[a] Spectroscopic data and apparent zinc dissociation constants ($K_{d\text{-}Zn}$) were determined in 25 mM PIPES buffer (pH 7) with 50 mM KCl and 50% (v/v) acetonitrile. [b]Quantum yield, absorption, and extinction coefficient values were taken from literature. [c]For comparison, the $K_{d\text{-}Zn}$ for TSQ was determined under identical conditions.

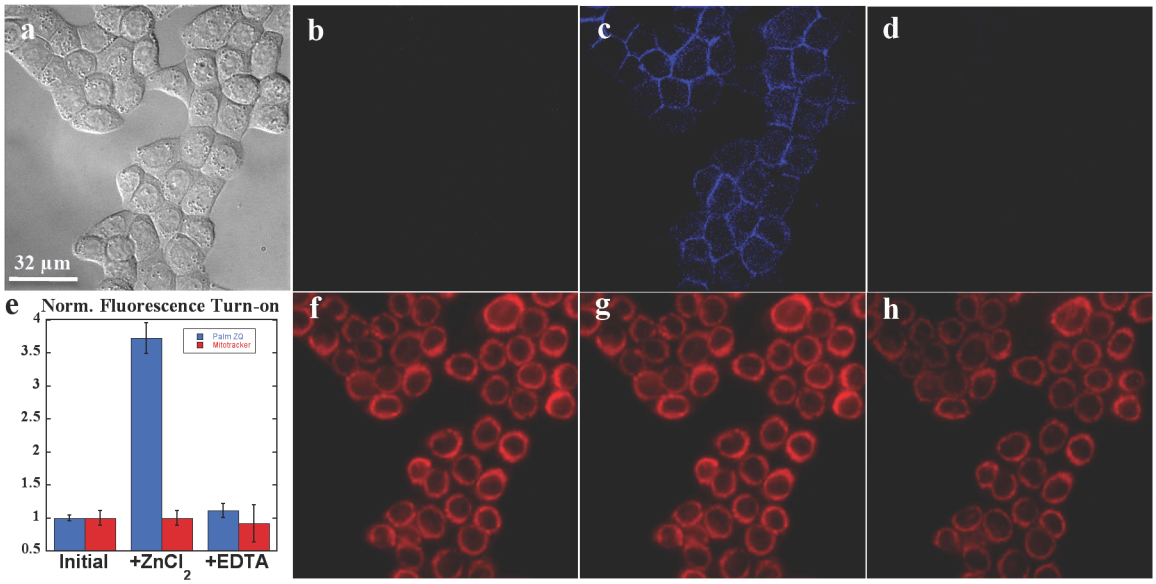

Figure S10. Zinc response of Palm-ZQ in live cell fluorescence imaging of RWPE-1 cells. (a) DIC Image. Signal intensity from Palm-ZQ, initially (b), after addition of 50 µM $ZnCl_2$ (c), and after addition of 100 µM EDTA (d). (e) Average normalized fluorescence signal of Palm-ZQ and Mitotracker red during live cell imaging. (n = 20). Signal Intensity from Mitotracker Red, initially (f), after addition of 50 µM $ZnCl_2$ (g), and after addition of 100 µM EDTA (h).

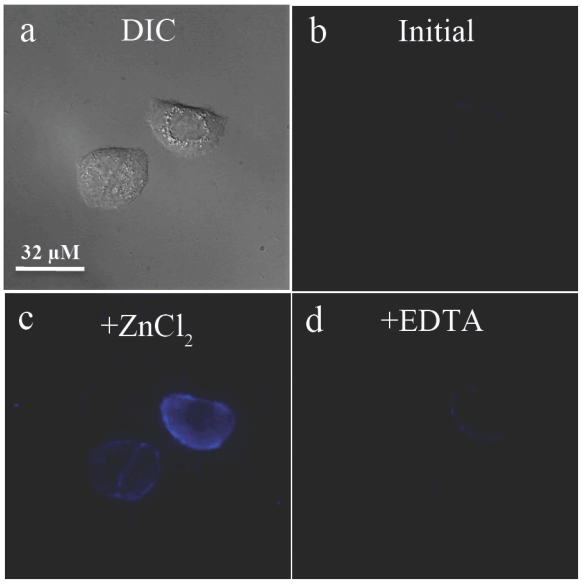

Figure S11. Zinc response of Palm-ZQ in live HeLa cells as monitored by fluorescence microscopy. (a) DIC. (b) Initial signal intensity from Palm-ZQ. (c) Emission after addition of 50 µM $ZnCl_2$. (d) Signal after addition of 100 µM EDTA.

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