Supporting Information for

An Endoperoxide Reactivity-Based FRET Probe for Ratiometric Fluorescence Imaging of Labile Iron Pools in Living Cells

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Experimental Details.

**General Methods, Cell Culture Procedures, and Confocal Fluorescence Imaging Experiments** are described in the article body.

**LC-MS Assays for Characterization of Fe(II) Reaction.** 100 µM FIP-1 was prepared in deoxygenated 10 mM HEPES (pH 7.4). Then ferrous ammonium sulfate (FAS) was added (from a 10mM stock in deoxygenated MilliQ water) to yield a final concentration of 1 mM FAS. This was stirred at 25 °C for 2.5 hours, then the sample was lyophilized overnight. The sample was reconstituted in MeOH, was filtered, then subjected to LC-MS analysis. LC-MS method was a linear gradient from 5 % MeOH / 95 % H₂O / 0.05 % formic acid to 95 % MeOH / 5 % H₂O / 0.05% formic acid over 50 minutes using an Agilent 300 extend-C18, 3.5 µm, 4.6 x 100mm column, monitoring UV at 254 nm. LRMS calcd. for 5-AMF-lactone C₃₂H₂₇NO₈ (Scheme 1) [M + H]⁺ 554.2, found 554.5. LRMS calcd. for Cy3-ketone C₃₅H₄₄N₃O₂⁺ (Scheme 1) [M]⁺ 538.3, found 538.4.

**Quantum Yield.** Quantum yield values were calculated using the equation \( \phi_{\text{sample}} = \phi_{\text{standard}} \frac{(\text{Grad}_{\text{sample}}/\text{Grad}_{\text{standard}})(\eta_{\text{sample}}^2/\eta_{\text{standard}}^2)} \) according to published methods.¹ Fluorescein prepared in 0.1 M NaOH was used as a standard, where \( \phi_{\text{standard}} = 0.92 \).² Quantum yield of the fluorescein-derived portion of FIP-1 was determined using linear unmixing with 5-AMF as the reference spectrum.

**FRET Efficiency.** FRET efficiency was assessed using the formula \( E = 1 - (\phi_{5\text{-AMF}} / \phi_{\text{FIP-1 fluor}}) \), where \( \phi_{\text{FIP-1 fluor}} \) is the quantum yield of the fluorescein-derived portion of FIP-1. Quantum yields were determined as described above.

**HEK 293T Fe(II) Dose Dependence Experiments.** DMEM culture media was aspirated from each chamber containing cells. This was replaced with 0 µM, 2 µM, 10 µM, or 100 µM FAS in DMEM media (without FBS and glutamax), and cells were incubated in this media for 60 minutes at 37 °C. After 60 minutes, DMEM media was aspirated and cells were washed 1x with 500 µL HBSS. Then 500 µL HBSS containing 10 µM FIP-1 (diluted from a 5 mM stock in DMSO) was added to each well and this was incubated at 37 °C for 90 minutes. At this point, buffer was removed and each well was washed 2x with 500 µL HBSS, then 500 µL of HBSS were added and snapshot images were taken.

**HEK 293T Chelator Dose Dependence Experiments.** 0, 10, 20, or 50 µM DFO was added to DMEM media containing 10% FBS and glutamax in chambers containing cells, and this was incubated at 37 °C for 8 hours. At this point, cells were washed 1x with 500 µL HBSS. Then 500 µL HBSS containing 10 µM FIP-1 (diluted from a 5 mM stock in DMSO) was added to each well and this was incubated at 37 °C for 90 minutes. At this point, buffer was removed and each well was washed 2x with 500 µL HBSS, then 500 µL of HBSS were added and snapshot images were taken.

**HEK 293T FIP-1 Dose Dependence Experiments.** DMEM culture media was aspirated from each chamber containing cells. This was replaced with either 0 µM or 10 µM FAS in DMEM media (without FBS and glutamax), and cells were incubated in this media for 60 minutes at 37 °C. After 60 minutes, DMEM media was aspirated and cells were washed 1x with 250 µL HBSS. Then 250 µL HBSS containing either 250 nM, 500 nM, 1 µM or 5 µM FIP-1 (diluted from a 1 mM stock in DMSO) was added to each well and this was incubated at 37 °C for 90 minutes. At this point, buffer was removed and each well was washed 2x with 250 µL HBSS, then 250 µL of HBSS were added and snapshot images were taken.
Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). HEK 293T cells were cultured in 6-well plates (Corning). 250 µM DFO or 1 mM BPS was added to DMEM media containing 10% FBS and glutamax in chambers containing cells and incubated at 37 °C for 8 hours. At this point, media in these wells was replaced with 250 µM DFO or 1 mM BPS containing DMEM media (without FBS and glutamax) and incubated for 90 minutes at 37 °C. DMEM media in non-treated wells was aspirated from chambers containing cells and this was replaced with DMEM media containing 100 µM FAS (prepared from a 20 mM FAS solution in water) or DMEM media and this was incubated for 90 minutes at 37 °C. The plates were then washed 2x with cold PBS containing 1 mM EDTA (to remove iron non-specifically bound to cell surfaces) then were washed 2x with cold PBS. 200 µL of concentrated nitric acid (BDH Aristar Ultra) was added to each well, then plates were sealed with parafilm (to prevent evaporation) and were allowed to sit at room temperature overnight. 150 µL of this digested solution was added to 2 mL of 2% solution of nitric acid in MilliQ water. Samples were analyzed on a Thermo Fisher iCAP Qc ICP mass spectrometer in Kinetic Energy Discrimination (KED) mode against a calibration curve of known iron and phosphorous concentrations. Values are reported as a ratio of $^{56}$Fe to phosphorous. Each condition was carried out in biological triplicate, and each experiment was performed in technical triplicate.

Flow Cytometric Analysis of HEK 293T Cell Viability using Propidium Iodide (PI) Staining. Cells were plated in 12-well polystyrene culture plates (Corning). 250 µM DFO or 1 mM BPS was added to DMEM media containing 10% FBS and glutamax in chambers containing cells and incubated at 37 °C for 8 hours. At this point, media in these wells was replaced with 250 µM DFO or 1 mM BPS containing DMEM media (without FBS and glutamax) and incubated for 90 minutes at 37 °C. DMEM media in non-treated wells was aspirated from chambers containing cells and this was replaced with DMEM media containing 100 µM FAS (prepared from a 20 mM FAS solution in water) or DMEM media and this was incubated for 90 minutes at 37 °C. After the incubation was complete, media was removed and 900 µL PBS containing 3 µM propidium iodide (PI) was added to each well. Cells were dislodged by agitation and filtered through 35 µm nylon mesh caps into a 12 x 75 mm polystyrene tube (Corning) for analysis by flow cytometry. Finally, cell viability was calculated as the percentage of PI negative cells.

Analysis of MDA-MB-231 Cell Viability using PrestoBlue Plate Reader Assay. MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone), glutamax (Gibco), and 1% non-essential amino acids (NEAA, Gibco). One day before the experiment, cells were passaged and plated in phenol-red free medium on clear-bottom, black 96-well plates at 4.5x10^4 cells/well. Media was aspirated and was replaced with 100 µL DMEM media containing 10% FBS and glutamax and either DMSO vehicle, 1.25 µM 35MEW28, 1.25 µM 35MEW28 + 1 µM ferrostatin-1 (Fer-1), or 1.25 µM 35MEW28 + 100 µM DFO at various time points. Cells were treated for 8, 10, 12, 16, 18 and 22 hours. At the end of the incubation period, compound-containing media was removed and was replaced with 100 µM DMEM media containing 10% PrestoBlue reagent (ThermoFisher). Cells were incubated for 1 hour at 37 °C, then fluorescence was read at 590 nm on a plate reader as per the package insert.

Generating Ratio Images. All quantification of images was done prior to generating ratio images. Ratio images were generated as following. Images were opened as 32-bit images in ImageJ then an ROI was selected in an area containing no cells. The background fluorescence measured in this area was subtracted from the image. A background subtraction was done on both the Green channel and on the FRET channel, then the Green channel was divided by the FRET channel using the Image calculator tool. For visualization, a median filter of 0.2 was applied.
Supplementary Figures.

Figure S1. UV-Visible spectra of FIP-1. (a) Spectra were obtained in 50 mM HEPES (pH 7.4) with 1 µM FIP-1 (dashed) and after (solid) treatment with 10 µM Fe(II).

Figure S2. Determination of molar attenuation coefficient at (a) λ = 488 nm and at (b) λ = 543 nm. Spectra were obtained in 50 mM HEPES (pH 7.4) with 1 µM FIP-1.
Figure S3. Determination of quantum yield for fluorescein portion of FIP-1 (circles) and for 5-AMF (triangles). The quantum yield for fluorescein portion of FIP-1 was determined to be 0.12 and the quantum yield for 5-AMF was determined to be 0.77. The quantum yield for the Cy3 derivatives is ca. 0.1.\(^5\)
Figure S4. (a) Kinetic profile of the reaction between 1 µM FIP-1 and 10 µM Fe(II) monitored at 515 nm. Reaction was carried out at 37 ºC in 50 mM HEPES (pH 7.4) with excitation at 488 nm. (b) Linearized integrated rate law, assuming this behaves as a pseudo first order reaction. $k_{obs} = 0.0016 \pm 0.00006$.

Figure S5. Dose dependence of 1 µM FIP-1 response to varying amounts of Fe(II). Data was acquired at 37 ºC in 50 mM HEPES (pH 7.4) with excitation at 488. Emission intensity was integrated over the range of 500-620 nm.

Figure S6. Dose dependence to Fe(II) addition in HEK 293T cells. HEK 239T cells were treated with 0 µM, 2 µM, 10 µM, or 100 µM FAS in serum-free DMEM media for 60 minutes at 37 ºC, then cells were washed and treated with 10 µM FIP-1 in HBSS for 90 minutes. Images were acquired after 2x HBSS washes. Error bars denote SEM, n=3. Statistical significance was assessed by calculating p-values using one-way ANOVA with the Bonferroni correction in R, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. 
Figure S7. Selectivity for Fe(II) over Cu(I) in HEK 293T cells. HEK 239T cells were treated with vehicle control (MilliQ water), 10 µM FAS, or 10 µM CuCl₂ in serum-free DMEM media for 90 minutes at 37 °C, then cells were washed and treated with 10 µM FIP-1 in HBSS for 90 minutes. Images were acquired after 2x HBSS washes. Error bars denote SEM, n=2.

Figure S8. FIP-1 responds to low dosages of DFO in HEK 293T cells. HEK 239T cells were treated with 0 µM, 10 µM, 20 µM or 50 µM DFO in DMEM media with 10% FBS and glutamax for 7 hours at 37 °C, then cells were washed and treated with 10 µM FIP-1 in HBSS for 90 minutes. Images were acquired after 2x HBSS washes. Error bars denote SD, n=2.
Figure S9. Total iron ($^{56}\text{Fe}$) content of control, chelator-treated and iron-supplemented HEK 293T cells. Chelator-treated HEK 293T cells were incubated with DMEM containing 250 µM DFO or 1 mM BPS and 10% FBS and glutamax for 8 hours at 37 °C, then were incubated for in serum-free DMEM containing 250 µM DFO or 1 mM BPS for 90 minutes at 37 °C. Iron-supplemented HEK 293T cells were incubated in serum-free DMEM containing 100 µM FAS for 90 minutes at 37 °C. Cellular $^{56}\text{Fe}$ and phosphorous content was measured by ICP-MS. Error bars denote SEM, n=3. Statistical significance was assessed by calculating p-values using one-way ANOVA with the Bonferroni correction in R, * $p < 0.05$.

Figure S10. FIP-1 Green/FRET ratio does not depend on FIP-1 concentration. HEK 293T cells were treated with either 0 µM or 10 µM FAS in DMEM media (without FBS and glutamax), and cells were incubated in this media for 60 minutes at 37 °C. After 60 minutes, DMEM media was aspirated and cells were washed 1x with 250 µL HBSS. Then 250 µL HBSS containing 500 nM, 1 µM, 5 µM, α10 µM FIP-1 (diluted from either a 1 mM or a 5 mM stock in DMSO) was added to each well and this was incubated at 37 °C for 90 minutes. At this point, buffer was removed and each well was washed 2x with 250 µL HBSS, then 250 µL of HBSS were added and snapshot images were taken. Error bars denote SEM, n=3.
**Figure S11.** Cell viability of HEK 293T cells upon chelator-treatment or iron-supplementation. Chelator-treated HEK 293T cells were incubated with DMEM containing 250 µM DFO or 1 mM BPS and 10% FBS and glutamax for 8 hours at 37 ºC, then were incubated for in serum-free DMEM containing 250 µM DFO or 1 mM BPS for 90 minutes at 37 ºC. Iron-supplemented HEK 293T cells were incubated in serum-free DMEM containing 100 µM FAS for 90 minutes at 37 ºC. Cell viability was measured by propidium iodide (PI assay). Error bars denote SEM, n=3.

**Figure S12.** Cell viability of HEK 293T cells upon chelator-treatment or iron-supplementation and after treatment with FIP-1. Chelator-treated HEK 293T cells were incubated with DMEM containing 250 µM DFO and 10% FBS and glutamax for 8 hours at 37 ºC, then were incubated for in serum-free DMEM containing 250 µM DFO for 90 minutes at 37 ºC. Iron-supplemented HEK 293T cells were incubated in serum-free DMEM containing 100 µM FAS for 90 minutes at 37 ºC. Media was removed and cells were treated for 90 minutes in HBSS containing 10 µM FIP-1, before washing 1x with HBSS. Cell viability was measured by propidium iodide (PI assay). Error bars denote SEM, n=2.
NMR Spectra.

5-(Methoxycarbonyl)-2-adamantanone, 2
Oxime ether 3
Endoperoxide 4
Endoperoxide-free amine 5
Endoperoxide-carboxylic acid 6
Cy3-NHS ester 8
Cy3-linker 7
References


