Supporting information for

A ratiometric iron probe enables investigation of iron distribution within tumour spheroids

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General Experimental Methods

All solvents used were laboratory grade and were used without further purification unless otherwise stated. Milli-Q water was used to prepare all aqueous solutions. Reagents were purchased from Sigma Aldrich, Alfa Aesar and Combi-blocks. Analytical thin layer chromatography was performed on commercially prepared silica plates (Merck Kieselgel 60, 0.25 mm F254). Flash column chromatography was performed using Davisil 230-400 mesh Kieselgel 60 silica eluting with solvents as described. Commercial materials were used as received unless otherwise noted.

Instrumentation

$^{1}$H NMR spectra were recorded at 300 K using a Bruker Avance DRX 300, Bruker Avance III 400 or 500. $^{13}$C NMR spectra were recorded on these spectrometers at a frequency of 75 or 100 MHz. NMR data are referenced to residual solvent signal and processed using Topspin (Bruker). Chemical shifts (δ) are expressed in parts per million (ppm). Abbreviations used to describe NMR spectra are: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and/or br (broad). Melting points were determined using a Digimelt MPA160. Low resolution mass spectrometry was performed on a Finnigan LCQ quadrupole ion trap mass spectrometer (EI) or a Bruker AmaZon SL ion trap mass spectrometer (ESI, APCI). High resolution ESI mass spectrometry was performed on a Bruker Apex qE 7T Fourier Transform Ion Cyclotron.

Fluorescence measurements were performed on a PerkinElmer Enspire Plate Reader or a CaryEclipse 4000 fluorometer. Selectivity experiments were performed with 0.1 or 10 equivalents of metal ions in HEPES buffer (20 mM, pH 7.4). Metals salts were delivered as LiCl, NaCl, KCl, MgCl$_2$, CaCl$_2$, MnCl$_2$, FeCl$_3$, Fe(NH$_4$)$_2$(SO$_4$)$_2$, Co(NO$_3$)$_2$, NiCl$_2$, Cu(NO$_3$)$_2$, [Cu(CH$_3$CN)$_4$]PF$_6$, and Zn(NO$_3$)$_2$. All fluorescence experiments were performed under aerobic conditions at room temperature.

Cellular Experiments

Cell culture

DLD-1 human colon carcinoma cells and RAW 264.7 mouse leukaemic monocyte macrophage cells were maintained in exponential growth as monolayers at 37 °C in 5% CO$_2$ in Dulbecco's modified eagle medium (DMEM) supplemented with 2.5 mM glutamine, 1% antibiotics, 10% fetal calf serum.
**Spheroid cell culture**

Agarose was prepared as a 0.75% w/w solution in PBS. Molten agarose (100 μl) was added to the wells of a 96 well plate. Agarose was allowed to cool before 1.0 x 10^5 cells were added to the well in 100 μl Advanced DMEM and allowed to grow for 4-5 days.

**Imaging experiments**

Cells were seeded in 35 mm MatTek dishes and grown to 60 - 90% confluence, at 37 °C in 5% CO₂. Cells were treated with a solution of appropriate concentration of probe in DMSO (final concentration of DMSO in cell media was less than or equal to 1%) and incubated for two hours before being washed with phosphate-buffered saline (PBS) and maintained in Live Cell Imaging Solution (140 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 20 mM HEPES, 10 mM D-glucose). For cultured cells, cells were incubated with salicylaldehyde isonicotinoyl hydrazone (10 μM) or ferrous ammonium sulfate (100 μM) for 30 min before imaging. For spheroid experiments, spheroids were incubated with deferiprone (100 μM) and deferasirox (100 μM) for 2 h before imaging.

**Confocal microscopy**

Confocal images were acquired using an Olympus FluoView FV1000 inverted light, fluorescence and confocal microscope and either a LUCPLFLN 40X air objective lens (NA = 0.60) or a UPLSAPO 60X water-immersion objective lens (NA = 1.20). Excitation light of 405 nm was provided by a LD405 nm laser and excitation at 488 nm was provided by a multi-line argon laser. Cells were imaged in a temperature-controlled incubator at 37 °C. Images were collected and processed using FV10-ASW viewer software v1.7 (Olympus). Image analysis of mean fluorescence intensity and z-stack projections were performed in FIJI/ImageJ (National Institutes of Health).
Scheme S1 Synthesis of FICFe1 and FICFe1'. Reagents and conditions: (a) EDCI, HOSu, DMF, rt, 48 h, 99%; (b) trans-1,4-diaminocyclohexane, DMF, rt, 24 h, 74%; (c) HOSu, DCC, DCM, rt, 40 min, 5a 29%, 5b 5%; (d) DMF, rt, 24 h, 23%; (e) K$_2$CO$_3$, MeOH, 30 °C, 16 h, then iminodiacetic acid, 9:1 MeOH/H$_2$O, 37% CH$_2$O$_{aq}$, 65 °C, 24 h, 81%; (f) Ac$_2$O, py, rt, 3 h, then
bromomethyl acetate, DIPEA, MeCN, rt, 15 h, 44%; (g) K₂CO₃, MeOH, 45 min, 0 °C to rt, 95%, or porcine liver esterase (1 mg/mL), 20 mM HEPES pH 7.4, 37 °C, 4 h.

Compounds 1, 2, 3¹ and 4² were prepared according to literature procedures.

5/6-(((2,5-Dioxopyrrolidin-1-yl)oxy)carbonyl)-3-oxo-3H-spiro[isobenzofuran-1,9’-xanthene]-3’,6’-diyl diacetate (5a and 5b)

To a stirring solution of 5/6-carboxyfluorescein diacetate (3.87 g, 8.41 mmol) in DCM (58 mL) was added N-hydroxysuccinimide (1.17 g, 10.2 mmol). N,N’-Diisopropylcarbodiimide (1.66 g, 13.2 mmol) was then added and the reaction stirred for 40 minutes. The mixture was washed with water (1 x 30 mL), brine (2 x 30 mL), the organic layer dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (25 to 100% EtOAc in toluene) to afford 5a (1.34 g, 29%) and 5b (225 mg, 5%) as white solids.

5a: Rᶠ (1:1 toluene/EtOAc) 0.35. ¹H NMR (300 MHz, CDCl₃) δ: 8.84 (s, 1H), 8.44 (d, J = 8.1 Hz, 1H), 7.38 (d, J = 8.1 Hz, 1H), 7.22 (d, J = 13.6 Hz, 2H), 7.16 (d, J = 2.0 Hz, 1H), 6.88 (dd, J = 8.7, 2.0 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 2.96 (s, 4H), 2.34 (s, 6H). ¹³C NMR (75 MHz, CDCl₃): 169.1, 168.8, 167.3, 160.6, 158.0, 152.5, 151.5, 137.8, 136.9, 129.0, 128.9, 128.2, 127.9, 127.6, 127.1, 125.3, 125.1, 118.1, 115.3, 110.7, 82.0, 77.6, 77.3, 77.0, 25.7, 25.6, 21.4, 21.0. LRMS (EI⁺): m/z 557 [M⁺].

5b: Rᶠ (1:1 toluene/EtOAc) 0.52. ¹H NMR (300 MHz, CDCl₃) δ: 8.42 (d, J = 8.0 Hz, 1H), 8.20 (d, J = 8.0 Hz, 1H), 7.95 (s, 1H), 7.23 (d, J = 14.0, 1H), 7.16 (d, J = 2.1 Hz, 1H), 6.89 (dd, J = 8.8, 2.1 Hz, 2H), 6.81 (d, J = 8.7 Hz, 2H), 2.91 (s, 4H), 2.34 (s, 6H). ¹³C NMR (75 MHz, CDCl₃): 168.8, 168.7, 167.5, 160.5, 153.0, 152.5, 151.5, 132.1, 131.6, 131.3, 129.0, 128.9, 128.2, 126.3, 126.3, 125.9, 125.3, 118.1, 115.2, 110.7, 82.3, 77.5, 76.8, 75.6, 21.4, 21.1.

5-((4-(7-(Diethylamino)-2-oxo-2H-chromene-3-carboxamido)cyclohexyl)carbamoyl)-3-oxo-3H-spiro[isobenzofuran-1,9’-xanthene]-3’,6’-diyl diacetate (6)

A solution of 5a (500 mg, 0.897 mmol) and 3 (296 mg, 0.827 mmol) in 15 mL DMF under nitrogen was stirred for 24 hours. The reaction mixture was then poured into a stirring ice/water slurry (125 mL). The resulting yellow precipitate was filtered and purified by silica gel column chromatography (0 to 70% EtOAc in toluene) to afford 6 (152 mg, 23%) as a pale yellow solid. Rᶠ (1:1 toluene/EtOAc) 0.19. ¹H NMR (300 MHz, CDCl₃) δ: 8.78 (d, J = 7.9 Hz, 1H), 8.71 (s, 1H), 8.35 (s, 1H), 8.20 (d, J = 8.0 Hz, 1H), 7.50 (d, J = 9.0 Hz, 1H), 7.20 (d, J = 7.5 Hz, 2H), 7.14 (s, 2H), 6.82 (t, J = 10.8 Hz 4H), 6.70 (d, J = 9.4 Hz, 1H), 6.53 (s, 1H), 6.17 (d, J = 7.9 Hz, 1H), 4.15-3.94 (m, 2H), 3.48 (q, J = 7.0 Hz, 4H), 2.34 (s, 6H), 1.61-1.24 (m, 14H). LRMS (ESI): m/z [M+Na]⁺ 822, [M–H]⁻ 798.
A solution of 6 (85 mg, 0.11 mmol) and K₂CO₃ (16 mg, 0.12 mmol) in 10 mL MeOH was stirred overnight at 30 °C. The reaction mixture was filtered and the filtrate evaporated under reduced pressure. The residue was dissolved in MeOH/H₂O (9:1, 6.5 mL), iminodiacetic acid (29 mg, 0.22 mmol) was added and the reaction mixture stirred at room temperature. Aqueous formaldehyde solution (37%, 0.4 mL) was then added dropwise, before the reaction mixture was heated at 65 °C for 24 hours. The solvent was then removed under reduced pressure to afford FlCFe1’ (87 mg, 81%) as an orange/red solid. ¹H NMR (300 MHz, DMSO-d₆): δ: 8.67 (s, 1H), 8.63 (d, J = 7.8 Hz, 1H), 8.56, (d, J = 7.5 Hz, 1H), 8.49 (s, 1H), 8.25 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 9.0 Hz, 1H), 7.38 (d, J = 7.8 Hz, 1H), 6.81 (dd, J = 9.0, 1.6 Hz, 1H), 6.69 (d, J = 1.6 Hz, 1H), 6.64 (s, 1H), 6.58-6.54 (m, 2H), 5.01-4.93 (m, 1H), 4.82-4.78 (m, 1H), 4.68 (s, 2H), 3.54-3.43 (m, 4H), 2.05-1.90 (m, 4H), 1.60-1.35 (m, 4H), 1.14 (t, 6H, J = 6.9 Hz).

Tetrakis(acetoxymethyl) 2,2',2'',2'''-(((3',6'-diacetoxy-5-((4-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)cyclohexyl)carbamoyl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-4',5'-diyl)bis(methylene))bis(azanetriyl))tetraacetate (FlCFe1)

A solution of FlCFe1 (20 mg, 0.02 mmol) and pyridine (20 µL, 0.25 mmol) in acetic anhydride (2 mL) was stirred for 3 h at 25 °C before the solvent was removed under a flow of nitrogen. The residue was dissolved in acetonitrile (5 mL), before N,N-diisopropylethylamine (30 µL, 0.17 mmol) and bromomethyl acetate (15 µL, 0.15 mmol) were added and the reaction mixture stirred at 25 °C under nitrogen for 15 h. The volatiles were then removed under reduced pressure before the residue was dissolved in EtOAc (10 mL) and washed with H₂O (10 mL). The aqueous layers were extracted with EtOAc (2 x 5 mL) and the combined organic layers washed with H₂O (2 x 5 mL). The organic layer was dried (Na₂SO₄) and the solvent removed by rotary evaporation. The residue was purified by preparative TLC (EtOAc) to afford FlCFe1 as an orange solid (10 mg, 44% yield). Rᵣ (EtOAc) 0.68. ¹H NMR (400 MHz, CDCl₃) δ: 8.82 (d, J = 7.8 Hz, 1H) 8.73 (s, 1H), 8.35 (s, 1H), 8.20, (dd, J = 7.8, 1.5 Hz, 1H), 7.46 (d, J = 9.0 Hz, 1H), 7.14 (d, J = 1.8 Hz, 1H), 6.84-6.82 (m, 3H), 6.67 (dd, J = 9.0, 2.4 Hz, 1H), 6.53 (d, J = 2.4 Hz, 1H), 6.12 (d, J = 7.5 Hz, 1H), 4.09-3.98 (m, 2H), 5.50-5.45 (m, 6 H), 5.40-5.36 (m, 12H), 3.48 (q, J = 7.2 4H), 2.34 (s, 8H), 2.27-2.18 (m, 4H), 2.13 (s, 6 H), 1.59-1.45 (m, 4H), 1.27 (t, 6H, J = 7.2 Hz). LRMS (ESI): m/z [M–(acetoxymethyl)₄+4H+3Na⁺] 386.1, [M–(acetoxymethyl)₄–(Ac)+6H⁺] 1048.2, [M–(acetoxymethyl)₄–(Ac)+3H⁻] 522.4.

References
**Fig. S1** Metal selectivity of FlCFe1’ towards (a) 1 µM metal ion and (b) 100 µM metal ion (10 µM probe, 20 mM HEPES pH 7.4, $\lambda_{ex} = 405$ nm, Ratio = integrated emission 420-485 nm / integrated emission 485-600 nm.
**Fig. S2** *FLICFe1* can report on exogenous changes in iron levels. Confocal microscope images ($\lambda_{ex} = 405$ nm) of DLD-1 cells treated with *FLICFe1* (10 µM, 2 h) followed by 30 min treatment with (i) vehicle control, (ii) salicylaldehyde isonicotinoyl hydrazone (SIH; 10 µM) or (iii) ferrous ammonium sulfate (FAS; 100 µM). (A) Blue channel (425-480 nm), (B) yellow channel (520-600 nm), (C) ratio (blue/yellow). Scale bars represent 20 µm.
Fig. S3 Confocal microscopy (488 nm excitation) of DLD-1 tumour spheroid (10^5 cells) incubated with calcein AM (10 μM, 24 h). Scale bar represents 200 μM.
Fig. S4 Confocal microscope images of (A) blue channel (425-480 nm), (B) yellow channel (520-600 nm) and (C) blue/yellow ratio image of DLD-1 spheroids (10⁶ cells) incubated with FLICFε1 (10 µM, 24 h) and then for 2 h with (i) vehicle control, (ii) deferiprone (100 µM) and (iii) deferasirox (100 µM). Scale bars represent 200 µm.
**Fig. S5** Fluorescence ratios at various distances into spheroids incubated with **FICFe1** (10 µM, 24 h) and subsequently treated for 2 h with vehicle control, deferiprone (100 µM) or deferasirox (100 µM). Error bars represent standard deviation of 3 independent experiments, *p*<0.05; **p**<0.01.
Fig. S6 Confocal microscope images of representative DLD-1 spheroids (10⁶ cells) incubated with calcein AM (10 µM, 24 h) and then for 2 h with (A) vehicle control, (B) deferiprone (100 µM) and (C) deferasirox (100 µM). Scale bars represent 100 µm. (D) Fluorescence intensities at various distances into spheroids. Error bars represent standard deviation of 3 independent experiments.